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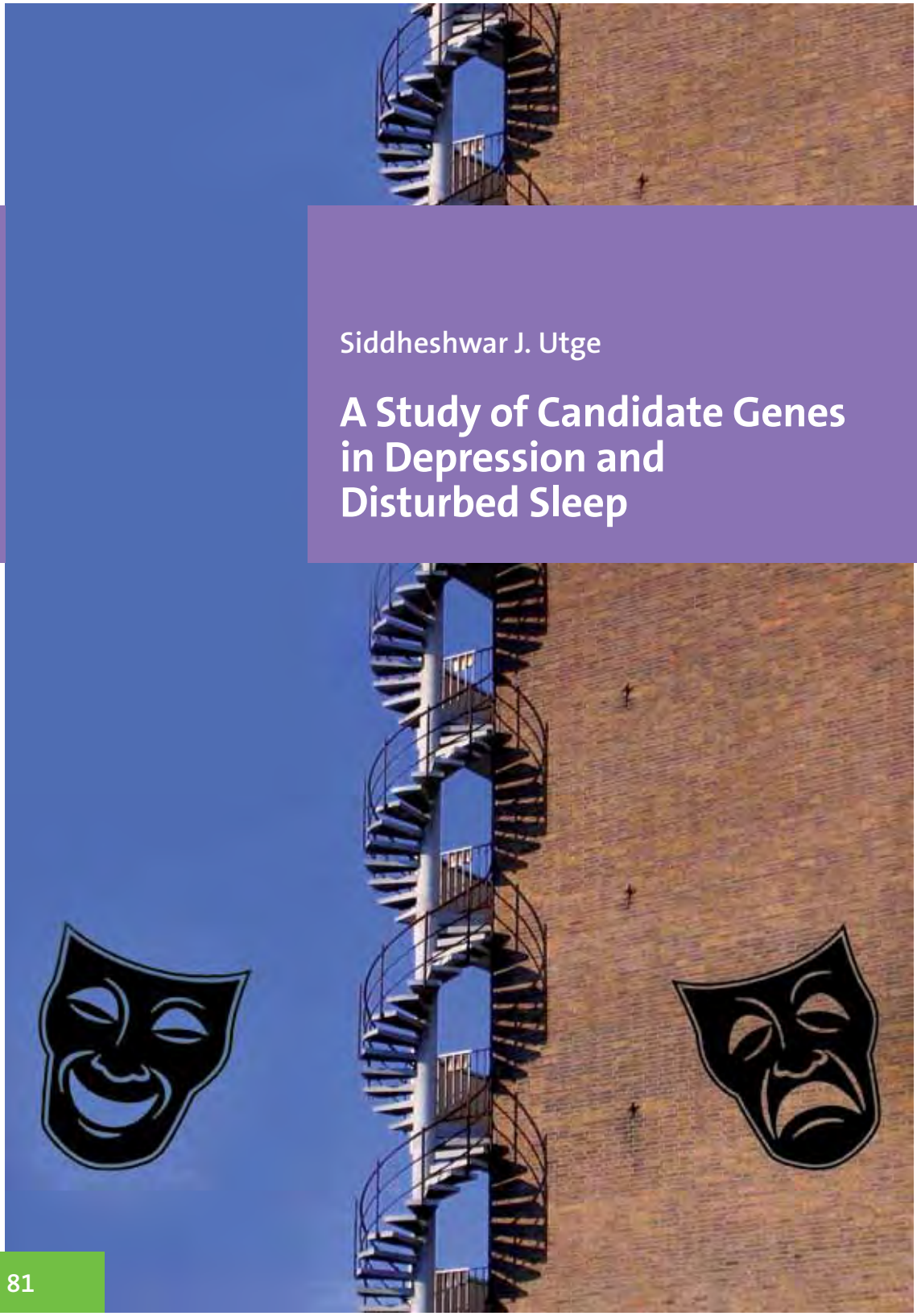

RESEARCH

Siddheshwar J. Utge

A Study of Candidate Genes in Depression and Disturbed Sleep

RESEARCH 81/2012

Siddheshwar J. Utge

A STUDY OF CANDIDATE GENES IN DEPRESSION AND DISTURBED SLEEP

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Medicine, University of Helsinki, for public examination in Lecture Hall2, Biomedicum Helsinki, on May 18, 2012 at 12.00.

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Helsinki 2012

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ISBN 978-952-245-650-2 (printed)
ISSN 1798-0054 (printed)
ISBN 978-952-245-651-9 (pdf)
ISSN 1798-0062 (pdf)

Juvenes print - Tampereen Yliopistopaino Oy
Tampere, Finland 2012

YMPÄRISTÖMERKKI
MILJÖMÄRKT
441 729
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To my family

Abstract

Siddheshwar J. Utge, a Study of Candidate Genes in Depression and Disturbed Sleep. National Institute for Health and Welfare (THL). Research 81/2012. 173 pages. Helsinki, Finland 2012.
ISBN 978-952-245-650-2 (printed); ISBN 978-952-245-651-9 (pdf)

Depression is a complex psychiatric disorder that comprises a variety of symptoms, in addition to depressed mood state, including indications of disturbed sleep such as early morning awakenings and fatigue. Poor sleep has been demonstrated to be one of the modifiable risk factors in the onset of depression. Neurobiological and molecular genetic studies of depression and disturbed sleep have introduced a number of candidate genes; the mechanism of genetic vulnerability for depressive disorders remains largely unknown, however. In the present study, depressive patients were grouped according to the presence or absence of disturbed sleep (e.g., early morning awakenings and fatigue). The genetic background of depression was hypothesized to be different between these groups. The regulation of sleep, and of mood, was assumed to partly share a common genetic background. The aim of this thesis was to identify genetic variants associated with depression and disturbed sleep in order to gain a better understanding of this hypothesis in the population-based Finnish samples.

First, the association between genetic markers from 14 functionally-relevant candidate genes was assessed. These genes were related to serotonergic and glutamatergic neurotransmission, to neural plasticity, and to the hypothalamic-pituitary-adrenal axis (HPA-axis) with depression, depression with early morning awakenings, and depression with fatigue in a sample from the population-based Health 2000 survey (http://www.terveys2000.fi/indexe.html). Overall, 1654 individuals were studied (384 depressed patients and 1270 population-matched controls). The data suggested that allelic variants from a gene coding for a key regulatory protein of the serotonergic neurotransmission system, namely tryptophan hydroxylase 2 (*TPH2*), is associated with depression accompanied by fatigue in females. An association between a number of genes related to glutamatergic neurotransmission and neural plasticity, such as glutamate decarboxylase 1 (*GAD1*), glutamate receptor, ionotropic, AMPA subunit 3 (*GRIA3*), and brain-derived neurotrophic factor (*BDNF*) with depression accompanied by fatigue in females. A significant association between cAMP responsive element binding protein 1 (*CREB1*), a neural plasticity related gene, and depression in men, was also identified. Of the genes related to the HPA-axis, an association was found between corticotropin releasing hormone receptor 1 (*CRHR1*) and depression accompanied by early morning awakenings in females.

The hypothesis was then expanded to encompass 18 genes from the circadian system in the same study subjects (N=1654) from the Health 2000 cohort. In this study, a significant association of two distinctive allelic variants of timeless homolog (Drosophila) (*TIMELESS*) was associated with depression accompanied by fatigue (Permutation-based corrected empirical P=0.0056), to seasonal mood fluctuation (Pointwise P=0.016) in females, and with depression accompanied by early morning awakenings (Permutation-based corrected empirical P=0.0374) in males. In an independent set of 1512 control individuals (Genmets (D-) sample) from the complete Health 2000 cohort, the same variant was also associated with seasonal mood fluctuation (Pointwise P=0.036) in females, and with early morning awakenings (Pointwise P=0.038) or fatigue (Pointwise P=0.0016) in healthy males.

Finally, the shared genetic background for sleep and mood in healthy individuals (N=3147) drawn from the population-based Health 2000 and FINRISK study 2007 survey (http://www.ktl.fi/portal/4168) was examined. In this study, for association analyses with sleep duration, 23 variants from 12 candidate genes were selected that had shown association (P<0.05) with depression and disturbed sleep in studies I and II. A significant association of a *GRIA3* variation with sleep duration in females (Permutation-based corrected empirical P=0.00001) was identified. The frequency of the allele which associated with depression was highest among females who slept for 8 hours or less in all age groups younger than 70 years. However, no prominent associations were found among males, suggesting a sex-specific effect for the X-chromosomal *GRIA3* gene.

In conclusion, these results support the involvement of genes, which are related to serotonergic (*TPH2*) or glutamatergic neurotransmission (*GAD1*, *GRIA3*), to neural plasticity (*BDNF*, *CREB1*), to the HPA-axis (*CRHR1*), and the circadian system (*TIMELESS*), in depression and disturbed sleep. The results obtained in this thesis support the hypothesis that the different phenotypes of depression and disturbed sleep would also be genetically distinct. The results indicate that depression is heterogeneous and that its genetic background may be partly different in women and men. This study also shows that the regulation of sleep and of mood may have common genetic backgrounds involving mechanisms of glutamatergic neurotransmission. Additional studies are warranted to investigate these findings further.

Keywords: depression, disturbed sleep, early morning awakenings, fatigue, candidate genes

Tiivistelmä

Siddheshwar J. Utge, a Study of Candidate Genes in Depression and Disturbed Sleep. [Tutkimus kandidaattigeenit masennus ja unihäiriöt]. Terveyden ja hyvinvoinnin laitos (THL). Tutkimus 81/2012, 173 sivua. Helsinki, Finland 2012.
ISBN 978-952-245-650-2 (printed); ISBN 978-952-245-651-9 (pdf)

Depressio on merkittävä psykiatrinen sairaus, johon kuuluu masentuneen mielialan lisäksi joukko muita oireita kuten unen häiriintymiseen liittyvät aamuyön heräilyt tai väsymys. Useat epidemiologiset tutkimukset osoittavat, että heikko uni on yksi merkittävimmistä depression riskitekijöistä. Jaottelimme tutkimuksessamme potilaat sen mukaan, ilmenikö heillä unen häiriöihin liittyviä oireita. Hypoteesinamme oli, että geneettinen depressioalttius olisi ainakin osin erilainen riippuen siitä, ilmeneekö potilaalla häiriintyneeseen uneen liittyviä oireita. Toinen tutkimushypoteesimme oli, että unen ja mielialan geneettisessä säätelyssä on ainakin osin yhteneväisiä mekanismeja.

Selvitimme aluksi toiminnallisten, serotoniini- tai glutamiinihermovälitykseen, plastisuuteen ja stressinsäätelyyn liityvien ehdokasgeenien assosiaatiota (i) depressioon, (ii) depressioon ja aamuyön heräilyyn ja (iii) depressioon ja väsymykseen väestöpohjaisessa Terveys 2000-aineistossa. Tuloksemme osoittivat että osa geeneistä, kuten serotoniinin tuottoa aivoissa säätelevä *TPH2*, liittyy depressioon naisilla silloin kun oireena on myös väsymystä. Lisäksi havaitsimme naisilla useiden eri glutamiinivälitykseen sekä plastisuuteen liittyvien geenien assosiaation depressioon ja väsymykseen ja stressinsäätelyjärjestelmän *CRHR1*-geenin assosiaation depressioon ja aamuyön heräämisiin. Miehillä totesimme depression merkittävän assosiaation *CREB1* – geeniin.

Laajensimme tutkimuksemme vuorokausirytmiä säätteleviin ns. kellogeeneihin. Havaitsimme kahden eri *TIMELESS*-geenimuodon assosioituvan depressioon ja väsymykseen sekä depressioon ja aamuyön heräämiseen miehillä. Samat riskivariantit asioituivat myös terveillä naisilla mielialan vuodenaikaiseen vaihteluun ja miehillä aamuyön heräilyihin.

Tutkimme myös depression ja mielialan geneettistä taustaa selvittämällä aiemmin tunnistamiemme riskivarianttien vaikutusta unen pituuteen terveillä, Terveys 2000 – ja Finriski 2007 – aineistoista poimituilla yksilöillä. Tunnistimme glutamiinireseptoria koodaavan *GRIA3*-geenin variaation assosiaation unen pituuteen. Sama alleeli, joka assosioitui naisilla depressioon, assosioitui terveillä naisilla lyhyeen yöuneen.

Tuloksemme osoittavat, kuinka serotoniini- (*TPH2*) ja glutamiinihermovälitykseen (*GAD1*, *GRIA3*), plastisuuteen (*CREB1*), HPA-akselin toimintaan (*CRHR1*) tai sir-

kadiaaniseen systeemiin (*TIMELESS*) liittyvillä geeneillä on tehtävä depression ja häiriintyneen unen etiologiassa. Tuloksemme viittaavat siihen, että depression etiologia olisi ainakin osittain erilainen riippuen potilaan oirekuvasta etenkin unen häiriön suhteen. Tuloksemme osoittavat myös depression heterogeenisyyden ja sen, että sairauden geneettinen etiologia on ainakin osittain erilainen naisilla ja miehillä. Lisäksi löydöksemme antavat viitettä siitä, että unen ja mielialan geneettinen säätely sisältävät päällekkäisiä, mm. glutamiinihermovälitykseen liittyviä mekanismeja. Jatkotutkimuksia tarvitaan, jotta voisimme ymmärtää näitä mekanismeja paremmin.

Avainsanat: depressioon, depressioon ja aamuyön heräilyyn, depressioon ja väsymykseen väestöpohjaisessa

Contents

List of original papers

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I **Utge S**, Soronen P, Partonen T, Loukola A, Kronholm E, Pirkola S, Nyman E, Porkka-Heiskanen T, Paunio T. 2009. A population-based association study of candidate genes for depression and sleep disturbance. *Am J Med Genet Part B*; 153B:468–476.

II **Utge SJ**, Soronen P, Loukola A, Kronholm E, Ollila HM, Pirkola S, Porkka-Heiskanen T, Partonen T, Paunio T. 2010. Systematic analysis of circadian genes in a population-based sample reveals association of *TIMELESS* with depression and sleep disturbance. *PLoS ONE*; 5(2): e9259. doi:10.1371/journal.pone.0009259.

III **Utge S**, Kronholm E, Partonen T, Soronen P, Ollila HM, Loukola A, Perola M, Salomaa V, Porkka-Heiskanen T, Paunio T. 2011. Shared genetic background for regulation of mood and sleep: association of *GRIA3* with sleep duration in healthy Finnish women. *SLEEP*; 34(10):1309–1316.

Abbreviations

A1R	Adenosine A1 receptors
A2A	Adenosine A2A receptors
ACTH	Adrenocorticotropic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
bp	Base pair
BP	Bipolar disorder
BF	Basal forebrain
cAMP	Cyclic adenosine monophosphate
CaMK	Calcium-calmodulin-dependent kinase
CEPH	Centre d'Etude du Polymorphisme Humain
CIDI	Composite International Diagnostic Interview
CNS	Central nervous system
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
CREB	cAMP responsive element binding protein
CRF/CRH	Corticotropin-releasing factor/hormone
CSF	Cerebrospinal fluid
D+	Patients with depression
D+EMA+	Depressed patients with early morning awakenings
D+FAT+	Depressed patients with fatigue
D+EMA+FAT+	Depressed patients with early morning awakenings and fatigue
D+EMA-FAT-	Depressed patients without early morning awakenings or fatigue
D+EMA+FAT-	Depressed patients with early morning awakenings but without fatigue
D+EMA-FAT+	Depressed patients without early morning awakenings but with fatigue
D-EMA-FAT-	Controls (no depression) without early morning awak enings or fatigue
D-	Controls (no depression)
D-EMA-	Controls without early morning awakenings
D-FAT-	Controls without fatigue
D-EMA+	Controls with early morning awakenings
D-FAT+	Controls with fatigue
DNA	Deoxyribonucleic acid

DOPAC	Dihydroxyphenylacetic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
DZ	Dizygotic
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
EMA	Early morning awakenings
ERα	Estrogen receptor alpha
GABA	γ-Aminobutyric acid
GATA-3	GATA binding protein 3
GxE	Gene-environment interaction
GR	Glucocorticoid receptor
GSS	Global seasonality score
GWAS	Genome-wide association studies
h	Hours
HapMap	Human Haplotype Map Project
HGP	Human Genome Project
HPA-axis	Hypothalamic-pituitary-adrenal axis
HVA	Homovanillic acid
HWE	Hardy–Weinberg equilibrium
kb	Kilobase
kDa	Kilodaltons
LD	Linkage disequilibrium
LOD	Logarithm of odds
LTP	Long-term potentation
LTD	Long-term depression
MAF	Minor allele frequency
MALDI-TOF	Matrix-assisted laser desorption-ionization-time of flight
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
M-CIDI	Munich-Composite International Diagnostic Interview
MDD	Major depressive disorder
MDE	Major depressive episode
mRNA	Messenger ribonucleic acid molecule
MRS	Magnetic resonance spectroscopy
MspI	Moraxella species I restriction enzyme
MZ	Monozygotic
NCBI	National Center for Biotechnology Information
NMDA	N-methyl-D-aspartate
NREM	Non-rapid eye movement
OR	Odds ratio
PCR	Polymerase chain reaction

PDE4	Phosphodiesterase 4
PET	Positron emission tomography
PKA	Protein kinase A
REM	Rapid eye movement
RFLP	Restriction fragment length polymorphism
RHT	Retino-hypothalamic tract
SCN	Suprachiasmatic nucleus
SCZ	Schizophrenia
SERT	Serotonin transporter
SNPs	Single nucleotide polymorphisms
SPAQ	Seasonal Pattern Assessment Questionnaire
TPH	Tryptophan hydroxylase
TRKB	Tyrosine kinase, receptor
UTR	Untranslated region
VNTR	Variable number of tandem repeat
WHO	World Health Organization
5-HTT	Serotonin transporter
$5\text{-HT}_{1A,\ 2A}$	Serotonin receptor A1, A2
5-HTTLPR	Serotonin transporter promoter variant

1 INTRODUCTION

Depression is a severe mood disorder, a common condition, and leading cause of morbidity and disability, affecting about 121 million people worldwide. Depression affects 6-7% of the population annually and 16% of people during their lifetime (Kessler *et al.*, 2003, Pirkola *et al.*, 2005). A recent study on a representative sample of the Finnish population (Health 2000) shows that depressive disorder is more common in women than in men; the respective 12-month prevalence rates are 8.3% and 4.6% (Pirkola *et al.*, 2005). According to twin studies, the heritability of major depressive disorder (MDD) is clearly higher in women (42%) than in men (29%), and some genetic risk factors are suggested to be involved (Kendler *et al.*, 2006). Recently, similar gender related findings were obtained for life dissatisfaction, depressed mood, and depressive disorder in a sample of 18,631 same-sex Finnish twins, with additive heritability estimates up to 53% in females and 39% in males (Paunio *et al.*, 2009).

Sleep problems are common in depression; about 90% of depressed individuals suffer from sleep difficulties (Tsuno *et al.*, 2005). People with depression have characteristic changes in their sleep architecture and show typically a poor quality of sleep, have trouble falling asleep, awaken frequently during the night, and also awaken very early in the morning with difficulties to get back to sleep (Benca *et al.*, 1997, Nofzinger *et al.*, 2004). As a consequence, they feel very tired or lacking in energy (fatigue) during the daytime (Benca *et al.*, 1997, Nofzinger *et al.*, 2004). Dysregulation of circadian rhythms also contributes to depressive episodes and to sleep disruption (McClung, 2007). Depression and disturbed sleep are complex processes, which involve various biological and psychological processes. The present study focuses on the correlation of genetic susceptibility factors, to depression with signs of disturbed sleep, such as early morning awakenings and fatigue.

The Human Genome Project (HGP) and the Human Haplotype Map Project (HapMap) have offered effective tools for the identification of genetic risk factors for etiologically multifactorial diseases. The work presented in this thesis explores genetic contributions to depression, and how they actually contribute to sleep variability. In this study, a candidate gene approach has been utilized to elucidate the genetic background of depression and disturbed sleep in Finnish population-based samples.

2 REVIEW OF THE LITERATURE

2.1 Overview of major depressive disorder

2.1.1 Diagnosis

Depression is characterized by depressed mood, accompanied by changes in thinking, feeling, behavior, and physical well-being. MDD consists of one or more major depressive episodes (MDE) lasting for two weeks with the symptoms presented in table 1 (American Psychiatric Association, 2000). According to the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR), the diagnosis of MDD requires at least five (or more) different symptoms, out of a list of nine, of which two are fatigue (or loss of energy) and insomnia (or hypersomnia). The patient's state represents a change from the individual's normal mood and performance. The symptoms must include depressed mood or a consistent loss of interest or pleasure in daily activities. A depressed mood due to the substances of drug abuse, to a medication or other treatment, or a general medical condition, is not considered to be MDD.

Table 1. Diagnostic criteria for major depressive episode as outlined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) (American Psychiatric Association, 2000).

A. Five of the following symptoms have been present during the same 2-week period and represent a change from previous functioning: at least one of the symptoms is either 1) or 2).
1) Depressed mood most of the day, nearly every day 2) Markedly diminished interest or pleasure in all, or almost all activities most of the day, nearly every day 3) Significant weight loss while not dieting, or weight gain, or decrease or increase in appetite nearly every day 4) Insomnia or hypersomnia nearly every day 5) Psychomotor agitation or retardation nearly every day 6) Fatigue or loss of energy nearly every day 7) Feelings of worthlessness or excessive or inappropriate guilt nearly every day 8) Diminished ability to think or concentrate or indecisiveness nearly every day 9) Recurrent thoughts of death, recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide
B. The symptoms do not meet criteria for a mixed episode.
C. The symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning.
D. The symptoms are not due to the direct physiological effects of a substance or a general medical condition.
E. The symptoms are not better accounted for by bereavement.

According to the DSM-IV-TR, dysthymia presents a chronic form of depression in which depressed mood is nearly constantly present for at least two years, accompanied by at least two (or more) of the other listed symptoms (Table 2) (American Psychiatric Association, 2000).

Table 2. Diagnostic criteria for dysthymia as outlined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) (American Psychiatric Association, 2000).

A. Depressed mood for most of the day, for more days than not, as indicated either by subjective account or observation by others, for at least 2 years.
B. Presence, while depressed, of two of the following: 1) Poor appetite or overeating 2) Insomnia or hypersomnia 3) Low energy or fatigue 4) Low self-esteem 5) Poor concentration or difficulty making decisions 6) Feelings of hopelessness
C. During the 2-year period of the disturbance, the person has never been without the symptoms of criteria A and B for more than 2 months at a time.
D. No major depressive episode has been present during the first 2 years of the disturbance; i.e., the disturbance is not better accounted for by chronic major depressive disorder, or major depressive disorder, in partial remission.
E. There has never been a manic episode, a mixed episode or a hypomanic episode, and criteria have never been met for cyclothymic disorder.
F. The disturbance does not occur exclusively during the course of a chronic psychotic disorder, such as schizophrenia or delusional disorder.
G. The symptoms are not due to the direct physiological effects of a substance or a general medical condition.
H. The symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning.

2.1.2 Epidemiology

2.1.2.1 Depression

Depression is anticipated to become a leading cause of disability worldwide by the year 2020 (Murray and Lopez, 1997). Epidemiological studies indicate that depression is highly prevalent in all populations (Kessler *et al.*, 1994, Kessler *et al.*, 2003, Jacobi *et al.*, 2004), but the estimates vary according to populations and studies. Prevalence is an epidemiological measure in public health; it is divided into point prevalence and period prevalence. The point prevalence is the percentage of people in a given population which suffer from a disorder or a specified condition at a point

in time. The period prevalence is the percentage of people having the condition during a given period of time, or a year (Streiner *et al.*, 2009). Lifetime prevalence is the percentage of people in a given population up to the time of assessment who have had the diagnosis of the disorder at some point in their life (Streiner *et al.*, 2009).

The life-time prevalence of MDD is 10-25% for females and 5-12% for males (American Psychiatric Association, 2000). The point prevalence of developing MDD at any given point in time is 5-9% for females and 2-3% for males (American Psychiatric Association, 2000). The prevalence rate of MDD varies worldwide, between an estimated life time prevalence of 3% in Japan, to 16.9% in the United States (Andrade *et al.*, 2003). Differences in these figures can arise, in principal, from differences in sampling, in populations and their racial/ethnic groups, from the diagnostic tools used, or from changes in prevalence over time within populations (Blazer *et al.*, 1994, Lindeman *et al.*, 2000). The various estimates for lifetime, point or period prevalence of major depression worldwide are summarized in table 3. Epidemiological studies on the general population show that the lifetime prevalence of dysthymia is 3-6% (Akiskal, 1994, Serretti *et al.*, 1999, Avrichir and Elkis, 2002). In the Finnish population, a 6-month prevalence of dysthymia was reported as 2% (Isometsa *et al.*, 1997) and the 12-month prevalence in the Health 2000 survey overall was 2.5% (3% for females and 1.9% for males) (Pirkola *et al.*, 2005).

Table 3. Epidemiological surveys on major depressive episode.

Country (Study)	*Diagnostic criteria*	*Prevalence (%)*	*Lifetime prevalence (%)*	*N*	*Reference*
Australia (NSMHWB)	CIDI (DSM-IV)	6.3 ***	n.s.	10641	(Andrews *et al.*, 2001)
Australia (NSMHWB)	CIDI (DSM-IV)	3.2*	n.s.	10641	(Wilhelm *et al.*, 2003)
Belgium (ESMeD)	CIDI (DSM-IV)	5.0***	14.1	2419	(Bromet *et al.*, 2011)
Brazil (ICPE)	WHO-CIDI (DSM-III-R)	3.9* 5.8***	12.6	1464	(Andrade *et al.*, 2003)
Brazil (Sao Paulo)	CIDI (DSM-IV)	10.4***	18.4	5037	(Bromet *et al.*, 2011)
Canada (ICPE)	UM-CIDI (DSM-III-R)	1.9* 4.3***	8.3	6902	(Andrade *et al.*, 2003)
Canada (CCHS1.2)	WMH-CIDI (DSM-IV)	1.8*	12.2	36984	(Patten *et al.*, 2006)
Chile (ICPE)	CIDI (DSM-III-R)	3.3* 5.6***	9.0	2978	(Andrade *et al.*, 2003)
China (Shenzhen)	CIDI (DSM-IV)	3.8***	6.5	7132	(Bromet *et al.*, 2011)
Colombia (NSMH)	CIDI (DSM-IV)	6.2***	13.3	4426	(Bromet *et al.*, 2011)
Czech Republic (ICPE)	CIDI (DSM-IV)	1.0* 2.0***	7.8	1534	(Andrade *et al.*, 2003)
Europe (DEPRES)	MINI (DSM-III)	6.9**	n.s.	13359	(Lepine *et al.*, 1997)
Finland (FINHCS)	UM-CIDI (DSM-III-R)	9.3***	n.s.	5993	(Lindeman *et al.*, 2000)
Finland	UM-CIDI (DSM-III-R)	4.1**	n.s.	2293	(Isometsa *et al.*, 1997)
Finland (Health 2000)	M-CIDI (DSM-IV)	4.9***	n.s.	6005	(Pirkola *et al.*, 2005)
France (ESMeD)	CIDI (DSM-IV)	5.9***	21.0	2894	(Bromet *et al.*, 2011)
Germany (ICPE)	M-CIDI (DSM-IV)	1.3* 5.2***	11.5	3021	(Andrade *et al.*, 2003)
Germany (ESMeD)	CIDI (DSM-IV)	3.0***	9.9	3555	(Bromet *et al.*, 2011)
India (WMHI)	CIDI (DSM-IV)	4.5***	9.0	2992	(Bromet *et al.*, 2011)
Israel (NHS)	CIDI (DSM-IV)	6.1***	10.2	4859	(Bromet *et al.*, 2011)
Italy (ESMeD)	CIDI (DSM-IV)	3.0***	9.9	4712	(Bromet *et al.*, 2011)
Japan (ICPE)	UM-CIDI (DSM-III-R)	0.9* 1.2***	3.0	1029	(Andrade *et al.*, 2003)
Japan WMHJ	CIDI (DSM-IV)	2.2***	6.6	3416	(Bromet *et al.*, 2011)
Korea (Seoul)	DSM-III	2.3***	2.9	5100	(Weissman *et al.*, 1996)
Lebanon (LEBANON)	CIDI (DSM-IV)	5.5***	10.9	2857	(Bromet *et al.*, 2011)

Mexico (ICPE)	UM-CIDI (DSM-III-R)	2.2* 4.5***	8.1	1734	(Andrade *et al.*, 2003)
Mexico (M-NCS)	CIDI (DSM-IV)	4.0***	8.0	5782	(Bromet *et al.*, 2011)
Netherlands (NEMESIS)	CIDI (DSM-III-R)	2.7* 5.8***	15.4	7076	(Bijl *et al.*, 1998)
Netherlands (ICPE)	WHO-CIDI (DSM-III-R)	2.7* 5.9***	15.7	7076	(Andrade *et al.*, 2003)
Netherlands (ESMeD)	CIDI (DSM-IV)	4.9***	17.9	2372	(Bromet *et al.*, 2011)
New Zealand	DSM-III	5.8***	11.6	1498	(Weissman *et al.*, 1996)
New Zealand (NZMHS)	CIDI (DSM-IV)	6.6***	17.8	12790	(Bromet *et al.*, 2011)
Puerto Rico	DSM-III	3.0***	4.3	1513	(Weissman *et al.*, 1996)
Spain (ESMeD)	CIDI (DSM-IV)	4.0***	10.6	5473	(Bromet *et al.*, 2011)
South Africa (SASH)	CIDI (DSM-IV)	4.9***	9.8	4315	(Bromet *et al.*, 2011)
Taiwan	DSM-III	0.8***	1.5	11004	(Weissman *et al.*, 1996)
Turkey (ICPE)	WHO-CIDI (DSM-III-R)	3.1* 3.5***	6.3	6095	(Andrade *et al.*, 2003)
Ukraine (CMDPSD)	CIDI (DSM-IV)	8.4***	14.6	4724	(Bromet *et al.*, 2011)
United States (ICPE)	UM-CIDI (DSM-III-R)	4.6* 10.0***	16.9	5877	(Andrade *et al.*, 2003)
United States (NCS-R)	CIDI (DSM-IV)	6.6***	16.2	9090	(Kessler *et al.*, 2003)
United States (NCS-R)	CIDI (DSM-IV)	8.3***	19.2	9282	(Bromet *et al.*, 2011)

N-Sample size,*point prevalence (a month or less), **6-month prevalence, ***12 month prevalence, n.s.-not specified. NSMHWB (National Survey of Mental Health and Well-being); ESMeD (The European Study of The Epidemiology of Mental Disorders); ICPE (International Consortium of Psychiatric Epidemiology); CCHS1.2 (The Canadian Community Health Survey: Mental Health and Well-Being); NSMH (The Colombian National Study of Mental Health); DEPRES (Depression Research in European Society); FINHCS (Finnish Health Care Survey); WMHI (World Mental Health India); NHS (Israel National Health Survey); WMHJ (World Mental Health Japan Survey); LEBANON (Lebanese Evaluation of the Burden of Ailments and Needs of the Nation); M-NCS (Mexican National Comorbidity Survey); NEMESIS (Netherlands Mental Health Survey and Incidence Study); NZMHS (New Zealand Mental Health Survey); SASH (South Africa Stress and Health Study); CMDPSD (Comorbid Mental Disorders during Periods of Social Disruption); NCS-R (National Comorbidity Survey Replication); UM-CIDI University of Michigan version of the Composite International Diagnostic Interview.

2.1.2.2 Sleep disturbances and depression

Most individuals suffering from depression experience disturbances, such as insomnia (Doghramji, 2003). In fact 90% of depressed individuals are reported to have sleep difficulties (Benca *et al.*, 1997, Tsuno *et al.*, 2005). Insomnia is a perception of insufficient sleep, and it affects approximately 9% to 12% of the population (Mellinger *et al.*, 1985, Ford and Kamerow, 1989, Taylor *et al.*, 2005). The lifetime prevalence of major depression has been reported to be 31.1% in individuals with a history of insomnia, 25.3% in those with hypersomnia, 54.3% in those with insomnia and hypersomnia, and 2.7% in those with no history of sleep disturbances (Breslau *et al.*, 1996).

Fatigue is one of the key symptoms of disturbed sleep in depression. The lifetime prevalence of depression among those with fatigue is 20.7% (Addington *et al.*, 2001), whereas individuals without a history of fatigue reported a 2.3% lifetime prevalence for depression (Addington *et al.*, 2001). Results of a cross-sectional study of depression in primary-care patients demonstrate that complaints of sleep disturbances and fatigue had the greatest predictive values (61% and 60%, respectively) for significant depressive symptoms (Gerber *et al.*, 1992). These epidemiological findings show that the individuals with sleep disturbances have an increased prevalence of major depression.

2.1.3 Etiology and candidate genes

Depression comprises a heterogeneous group of disorders (Kendler *et al.*, 1993). The etiology of depression includes psychological, physiological, and environmental factors (Bebbington, 1999, Manji *et al.*, 2001, Caspi *et al.*, 2003, Kendler *et al.*, 2004), as summarized in figure 1.

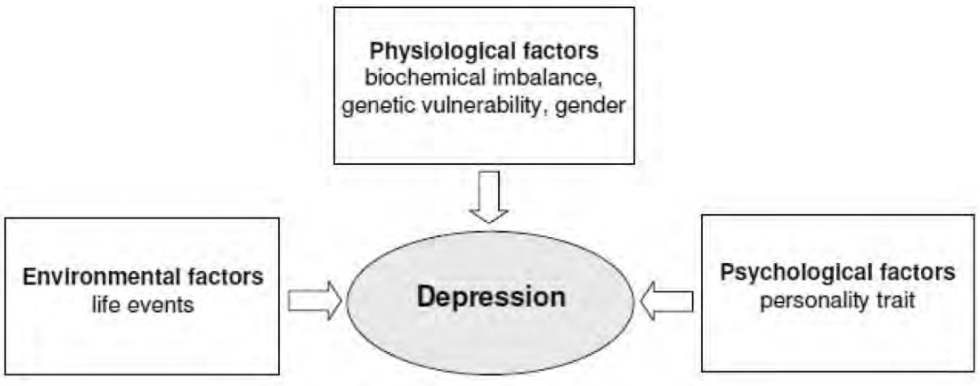


Figure 1. Etiological factors considered to contribute to depression.

Neurotransmitters

Neurotransmitters are chemical messengers which conduct signals from one nerve cell to another. They play a central role in the pathophysiology of brain disorders, thus are targets for pharmacological treatments. Among the various neurotransmitters and their roles, the present thesis is focusing on the monoamines, particularly serotonin, amino acids (glutamate), and neuropeptide neurotransmitters (corticotropin releasing hormone), which will be discussed in more detail in the following sections.

Monoamines

It has been hypothesized, that reduced levels of monoamines at the synapse underlie depression or mood disorders (Castren, 2005). This theory is supported by the clinical finding that monoamine re-uptake inhibitors, which increase the neurotransmitter level in the synaptic cleft, alleviate the symptoms of depression (Castren, 2005).

***Serotonin*.** Serotonin is synthesized from tryptophan. In the human body, most serotonin is found in the enterochromaffin cells of the digestive tract where it regulates intestinal movements (Reid and Rand, 1952). In the brain, most serotonergic neurons are located in the raphe nuclei (Figure 2) (Berger *et al.*, 2009).

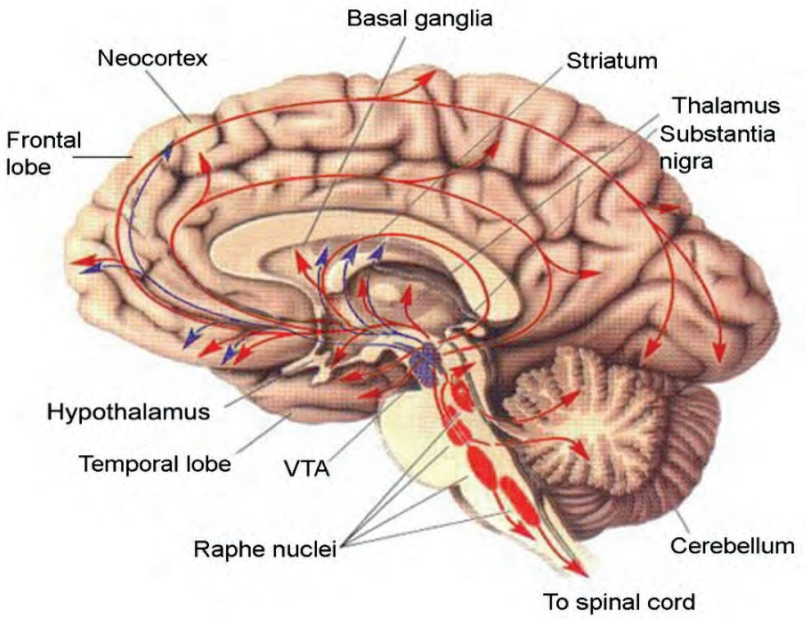


Figure 2. Serotonin and dopamine pathways in the brain. A serotonergic pathway (red) arises from neurons in the monoaminergic cell groups, including the dorsal and median raphe nuclei which are grouped together in the brainstem (Saper *et al.*, 2005). A dopaminergic pathway (blue) arises from neurons in the monoaminergic cell groups, including the substantia nigra and ventral tegmental areas (VTA) which are located in the midbrain (Stahl and Mignon, 2010).

Elevated or decreased levels of serotonin in the brain may also cause drastic changes in behavior (Saper *et al.*, 2005), and this neurotransmitter system has accordingly been widely investigated in the pathophysiology of depression, or psychiatric disorders (Manji *et al.*, 2001). Many components of the serotonergic system, such as serotonin-1a receptor (5-HT_{1A}), serotonin-2a receptor (5-HT_{2A}) and serotonin transporter (5-HTT), are associated with the neurobiology of mood and act as mediators for the therapeutic actions of antidepressants (Rajkumar and Mahesh, 2008).

A low cerebrospinal fluid (CSF) level of the serotonin metabolite 5-hydroxyindolacetic acid (5-HIAA) is associated with depression (Placidi *et al.*, 2001). *In vivo* imaging studies of depressed patients and controls show differences in the binding of 5-HTT in the regions that receive dense serotonergic projections from the midbrain raphe nuclei. Lower 5-HTT binding in the amygdala and midbrain region in patients with major depression has been found in positron emission tomography (PET) studies (Parsey *et al.*, 2006, Oquendo *et al.*, 2007). These find-

ings indicate that the function of 5-HTT contributes to the pathogenesis of depression.

***Catecholamines*.** The family of catecholamines includes adrenaline (A) (epinephrine), noradrenaline (NA) (norepinephrine), and dopamine, all synthesized from tyrosine. In the CSF, the concentration of NA has been reported to be low in depressed patients (Post *et al.*, 1978), while in some studies it has been reported to be higher in the plasma of depressed patients (Esler *et al.*, 1982, Rothschild *et al.*, 1987, Kelly, 2004). However, not all studies report elevated plasma levels (Rudorfer *et al.*, 1985, Healy *et al.*, 1992). Dopamine is synthesized in cell groups of the substantia nigra and the VTA in the midbrain (Figure 2). Dysfunction in dopaminergic neurotransmission has been suggested in the pathophysiology of depression, since dopamine metabolites have been measured in the plasma of depressed patients. Several studies consequently reported that the plasma levels of the dopamine metabolite (HVA) were lower in depressed patients (Mitani *et al.*, 2006a). In contrast, suicide victims without a history of depression have normal levels of HVA, dopamine, and NA, based on the results of some autopsy studies (Pare *et al.*, 1969, Beskow *et al.*, 1976).

Several genes and proteins are involved in the regulation of the monoaminergic pathways (Figure 3). Genetically determined alterations in their activity may thus have a role in the vulnerability for depression. The present thesis focused on the candidate genes summarized in table 4.

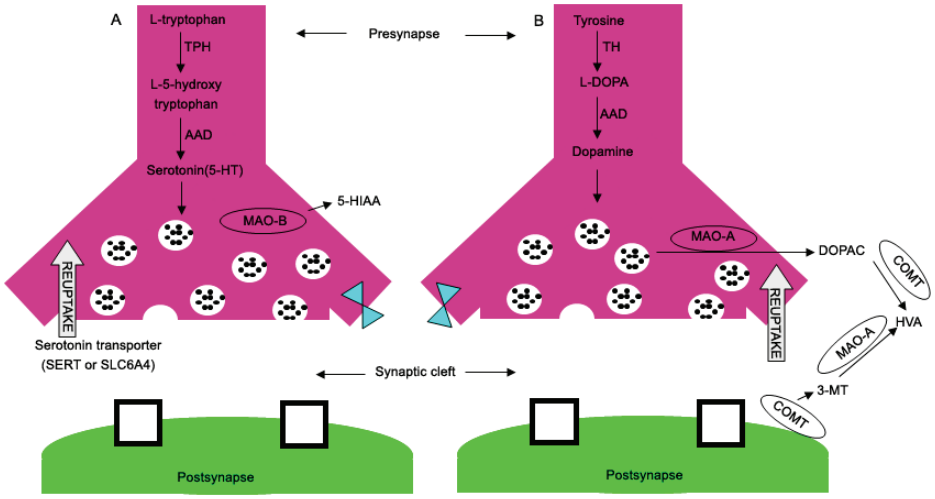


Figure 3. Schematic overview of serotonin and dopamine neurotransmission. Neurotransmission consists of several steps, including: 1) synthesis of neurotransmitters, 2) storage in synaptic vesicles, 3) release into the synaptic cleft upon depolarization of the neuron, 4) binding to postsynaptic receptors or binding to autoreceptors located on the presynaptic membrane, 5) reuptake from the synaptic cleft after depolarization (Mohammad-Zadeh *et al.*, 2008). Panel A) The synthesis, storage, release, and uptake of serotonin via the selective serotonin transporter (*5-HTTLPR* variant of SERT/SLC6A4), and its metabolism via MAO-B. Panel B) The synthesis and metabolism of dopamine (Catecholamine). Dopamine is metabolized by monoamine oxidase-A (MAO-A) to 3, 4-dihydroxyphenylacetic acid (DOPAC). DOPAC diffuses out of the presynapse and transformed to homovanillic acid (HVA) in the presence of catechol-O-methyltransferase (COMT) (Westerink, 1985, Mannisto *et al.*, 1992). COMT is located outside the dopaminergic neurons. A fraction of the released dopamine is metabolized to 3-methoxytyramine (3-MT), then oxidized in the presence of MAO-A to HVA (Westerink, 1985, Mannisto *et al.*, 1992). TPH=Tryptophan hydroxylase, AAD=Amino acid decarboxylase, MAO-B=monoamine oxidase-B, 5-HIAA=5-hydroxyindoleacetic acid. TH=Tyrosine hydroxylase, L-DOPA=levodopa.

Table 4. Candidate genes involved in the regulation of serotonin and catecholamine.

Gene	*Name*	*Function*	*Chromosome*	*Reference in depression*
TPH2	Tryptophan hydroxylase 2	Rate-limiting enzymes involved in the biosynthesis of serotonin	12q21	(Zill *et al.*, 2004a)
MAOA	Monoamine oxidase A	Degrades amine neurotransmitters	Xp11.3	(Schulze *et al.*, 2000)
SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	Serotonin transporter	17q11-q12	(Ogilvie *et al.*, 1996)
COMT	catechol-O-methyltransferase	Role in the breakdown of dopamine in brain	22q11.21	(Massat *et al.*, 2005)

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme for the synthesis of serotonin (Figure 3). TPH determines the amount of serotonin available in the brain. Therefore, it is regarded as a candidate gene for the pathogenesis of depression. It has two isoforms, TPH1 and TPH2 (Mockus and Vrana, 1998). *TPH1* gene is located on chromosome 11p15.3–p16 and *TPH2* on chromosome 12q15, spans a region of 29 kb and 93.5 kb, respectively. Both are highly homologous and share 71% of their amino-acids (Walther and Bader, 2003). TPH2 is a brain-specific isoform, and acts as rate-limiting enzyme in the synthesis of serotonin in neural tissue (Walther and Bader, 2003). The peripheral biosynthesis of serotonin is initiated by TPH1 (Walther and Bader, 2003). Studies on human and rodent tissues confirmed that TPH1 is predominantly expressed in the enterochromaffin cells of the gut and in lower amounts in the pineal gland (Brain) (Sakowski *et al.*, 2006). TPH2 is not expressed in peripheral organs (liver, kidney, heart or lung), but mainly expressed in the neurons of the raphe nuclei in the brain stem, and in peripheral myenteric neurons in the gut (Matthes *et al.*, 2010). Several genetic studies have reported an association with single nucleotide polymorphisms (SNPs) in *TPH2* and depressive disorder (Zill *et al.*, 2004a, Zhang *et al.*, 2005, Zhou *et al.*, 2005, Van Den Bogaert *et al.*, 2006, Haghighi *et al.*, 2008, Tsai *et al.*, 2009), or with suicide in MDD (Zill *et al.*, 2004b, Ke *et al.*, 2006, Lopez de Lara *et al.*, 2007).

Monoamine oxidase (MAO) is a mitochondrial outer membrane-bound enzyme involved in the degradation of serotonin and dopamine (Figure 3). MAO exists in two forms: MAO-A and MAO-B. Both the genes encoding the MAOs are located on the X-chromosome and are homologous; they share 60% identity (Zhu *et al.*, 1992). In the CNS, MAO-A is found in the catecholaminergic neurons (Lewis *et al.*, 2007), while MAO-B is highly expressed in the serotonergic neurons, and astrocytes (Chen, 2004). MAO enzymes preferentially degrade serotonin, catecholamines by oxidative deamination (Shih and Thompson, 1999), an ability which made these enzymes candidates for the study of neurological diseases, and of psychiatric and behavioural phenotypes. There is variable number tandem repeat (VNTR) functional polymorphism in the promoter region of *MAO-A* gene. This polymorphic sequence consists of a 30-bp repeated sequence (*MAO-A-30 bp VNTR*) as 2, 3, 3.5, 4 or 5 repeats (R) and has been shown to affect the transcriptional activity of the *MAO-A* promoter (Sabol *et al.*, 1998). The alleles with (3.5R or 4R) of the repeat sequence are transcribed more efficiently than those with (3R or 5R) copies of the repeat (Sabol *et al.*, 1998). Therefore, the alleles with 3.5R or 4R are related to higher central MAO-A activity and to lower central serotonin level (Sabol *et al.*, 1998). An association study of this functional polymorphism showed the frequency of the 4R allele significantly increased in MDD patients (Yu *et al.*, 2005).

The serotonin transporter gene 5-HTT (*SLC6A4*) is located on the chromosome 17q11.2, spanning a 38 kb region. The variation (5-HTT -linked polymorphic region, *5-HTTLPR*) at the promoter region of *SLC6A4* modulates the transcriptional activity of the gene. The *5-HTTLPR* consists of two common alleles, a 44-bp insertion or 16 imperfect repeats of an approximately 22-base-pair element (long or L allele), and a deletion or allele with 14 repeats (short or S allele) located exactly at the 5'-flanking regulatory region (Heils *et al.*, 1996). The S allele of *5-HTTLPR* relate to significantly less mRNA and protein, resulting in a lower concentration of serotonin in the synaptic cleft, than the L variant (Lesch *et al.*, 1996). The S allele of *5-HTTLPR* is associated with increased risk for depression in the presence of stressful life events, such as childhood maltreatment (Caspi *et al.*, 2003). The promoter sequence within the *5-HTTLPR* region also includes a single nucleotide polymorphism (SNP) rs25531 (A→G substitution) (Nakamura *et al.*, 2000), where the G allele diminishes *SLC6A4* transcription efficiency (Hu *et al.*, 2006). Other polymorphic regions in the serotonin transporter include a 17 bp variable number of tandem repeats in the second intron (intron 2 VNTR or STin2 VNTR) which also modulates the transcription of *SLC6A4* (Hranilovic *et al.*, 2004). Genetic variation in the serotonin transporter gene (*5-HTTLPR*, rs25531) associated with an increased risk for MDD (Holmes *et al.*, 2010).

The catechol-o-methyltransferase (*COMT*) gene is located on the chromosome 22q11.21, spanning a 27.2 kb region. The enzyme catechol-o-methyltransferase

degrades catecholamines and helps to maintain their level in the brain. The most widely studied variation within the *COMT* gene is the Val108/158Met polymorphism (rs4680) which changes the amino acid valine to methionine at position 108 of the shorter form of the enzyme or at position 158 of the longer form (Williams *et al.*, 2007). This variation within the *COMT* gene is associated with various mental illnesses (Craddock *et al.*, 2006).

Amino acids (Glutamate)

Glutamate is the excitatory amino acid neurotransmitter which is ubiquitously distributed in mammalian brain tissue (Fonnum, 1984). There is growing evidence that dysregulation of glutamate occurs in patients with MDD (Mitchell and Baker, 2009). Increased glutamate levels were measured in the plasma of depressed patients (Mauri *et al.*, 1998, Mitani *et al.*, 2006b). The CSF level of glutamate in patients with MDD was also significantly higher than in controls (Levine *et al.*, 2000). *Post-mortem* studies have shown that brain glutamate levels were elevated in the frontal cortex tissues of depressed patients (Hashimoto *et al.*, 2007, Feyissa *et al.*, 2010), whilst a magnetic resonance spectroscopy (MRS) study in patients with MDD revealed increased levels of glutamate in the occipital cortex (Sanacora *et al.*, 2004). Autopsy studies showed lower mRNA levels of glutamate receptors (NMDA, AMPA and kinate) in tissue samples from the prefrontal cortex of patients with MDD (Beneyto *et al.*, 2007, Feyissa *et al.*, 2009). In addition, to date, evidence has emerged that glutamatergic receptors have antidepressant-like activity (Paul and Skolnick, 2003).

Several proteins and genes are involved in glutamatergic neurotransmission. The present study addresses the following candidate genes (Figure 4; Table 5) which have, in previous studies, shown to be involved in the etiology of neuropsychiatric disorders.

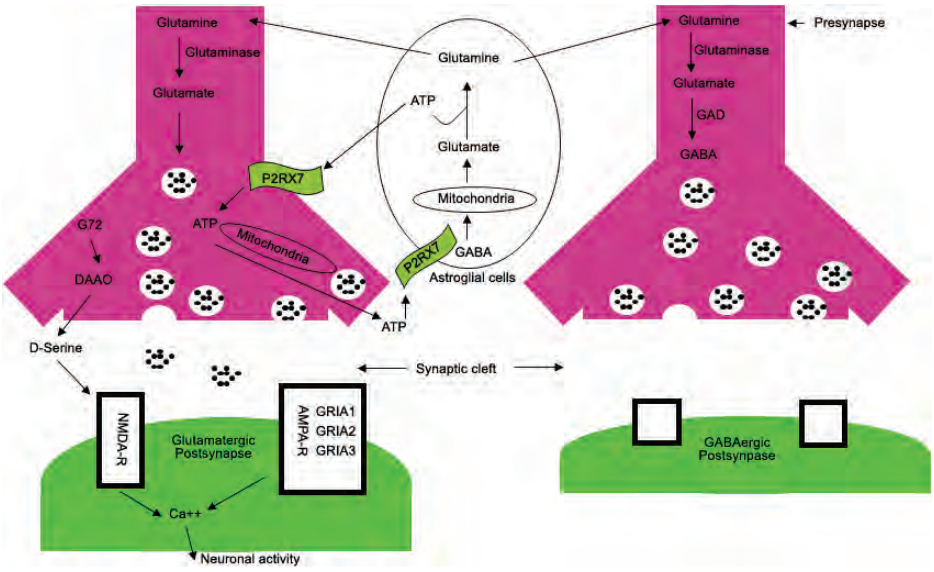


Figure 4. Schematic overview of glutamate synthesis and metabolism. In the brain, glutamate is generated as a by-product of energy metabolism and plays a role in all known brain functions. The metabolism of glutamate involves both neurons and astroglial cells. In astroglial cells, the γ-Aminobutyric acid (GABA) and glutamate are metabolized to glutamine and recycled to neurons to form glutamate or GABA (Palmada and Centelles, 1998). Released adenosine triphosphate (ATP), coenzyme in astroglial cells enters into the glutamatergic presynapse via purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7) (Illes and Ribeiro, 2004). ATP promotes calcium ion (Ca^{++}) influx into the presynaptic terminal and leads to the release of glutamate from the presynaptic neurons (Burnstock, 2008). Released glutamate binds to N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors or kainate receptors (Mitchell and Baker, 2009). The normal function of D-serine in the brain is to activate the NMDA receptor (Chumakov *et al.*, 2002). GABA is an inhibitory neurotransmitter, synthesized from glutamate with the help of the enzyme glutamic acid decarboxylase (GAD). The excess amount of glutamate, GABA and ATP is taken up by surrounding astroglial cells. GRIA1=glutamate receptor, ionotropic, AMPA subunit 1, GRIA2=glutamate receptor, ionotropic, AMPA subunit 2, GRIA3=glutamate receptor, ionotropic, AMPA subunit 3.

Table 5. Candidate genes involved in the regulation of glutamate.

Gene	*Name*	*Function*	*Chromosome*	*Reference in depression*
DAOA (G72)	D-amino acid oxidase activator	Glutamate signaling	13q33	(McGuffin *et al.*, 2005, Rietschel *et al.*, 2008)
GAD1	Glutamate decarboxylase 1	Catalyzes production of GABA	2q31.1	(Hettema *et al.*, 2006)
GRIA3	Glutamate receptor, ionotropic, AMPA subunit 3	Glutamate receptor	Xq25	(Laje *et al.*, 2007)
P2RX7	Purinergic receptor P2X, ligand-gated ion channel, 7	ATP binding, ion channel activity	12q24	(Lucae *et al.*, 2006)

D-amino acid oxidase activator (*DAOA*) (or named *G72*) is located on chromosome 13q33, spanning a 65 kb region. The G72 protein activates the enzyme D-amino acid oxidase (DAAO), which oxidizes D-serine (Boks *et al.*, 2007) (Figure 4), and consequently attenuates glutamatergic neurotransmission at NMDA receptors (Hall *et al.*, 2008). The genetic marker from *G72* is found to be associated with bipolar disorder, schizophrenia (SCZ), and depression (Hall *et al.*, 2008, Soronen *et al.*, 2008, Rietschel *et al.*, 2008).

Glutamate decarboxylase (*GAD*), is the rate-limiting enzyme in the conversion of glutamate to GABA in the mammalian brain (Harrison, 2007). The activation of GABA (A) receptors favors sleep (Gottesmann, 2002). GAD has two isoforms, *GAD1* and *GAD2* (Fehr *et al.*, 2003), located on chromosomes 2q31.1 and 10p11.23, spanning 45 kb and 79 kb genomic regions, respectively (Bu and Tobin, 1994). The genes *GAD1* and *GAD2* encode proteins with molecular weights of 65 kDa (GAD_{65}) and 67 kDa (GAD_{67}), which are coexpressed in GABAergic neurons. A *postmortem* brain study showed a reduction in the levels of GAD proteins in the cerebellum of patients with schizophrenia, bipolar disorder, and major depression (Fatemi *et al.*, 2005).

The glutamate receptor, ionotropic, AMPA subunit 3 (*GRIA3*) is one of the four AMPA receptor subunits. *GRIA3* gene is located on chromosome Xq25, spanning a 300 kb genomic region, and mediates the signaling of excitatory neurotransmission in the brain (Gecz *et al.*, 1999). *GRIA3* is expressed in the reticular nucleus of the thalamus and the cortex (Beneyto and Meador-Woodruff, 2004), which are important areas in the regulation of sleep and wakefulness (McCormick and Bal, 1997, Steriade, 2006).

The purinergic receptor P2X, ligand-gated ion channel, 7 (*P2RX7*), a gene coding for a purinergic ligand-gated ion channel, belongs to the P2RX receptor family. *P2RX7* is located on the chromosome 12q24, spanning a 250 kb region. This gene is activated by an increased level of extracellular ATP (Surprenant *et al.*, 1996) (Figure

4), and has a role in learning and memory responses, and in mood disorders (Burnstock, 2008, Soronen *et al.*, 2011).

Neuroplasticity

In the biological sciences, the term plasticity refers to the capability of cells or organisms to change their phenotypes in response to their environment (Skipper *et al.*, 2010). It specifically refers to the ability of neurons to acquire information based on outside experiences and make the appropriate responses by strengthening or weakening nerve connections (synapses), or by adding new nerve cells and forming new synaptic connections to compensate for injury (Rao *et al.*, 1999, Rioult-Pedotti and Donoghue, 2003). Neuroplasticity is involved in encoding information during learning and memory storage (Duman *et al.*, 2000). There are several studies indicating that impairment of neuroplasticity may be involved in the development of many neuropsychiatric diseases (Teter and Ashford, 2002, Frost *et al.*, 2004, Castren, 2005).

Patients with major depression exhibit memory problems, such as a diminished ability to think or to concentrate. Hippocampal activity is essential for cognitive functions (Zakzanis *et al.*, 1998). An experimental animal study showed that chronic or severe stress can disrupt hippocampus-dependent memory (Diamond *et al.*, 1999). Stress-induced deficits in hippocampus-dependent memory formation are observed in depression (Pittenger and Duman, 2008). Depressed patients show a volume loss in their hippocampus, possibly due to glutamate neurotoxicity-induced tissue loss, stress-induced reduction in neurotrophic factors or stress-induced reduction in neurogenesis (Sheline, 2000). Hippocampal synaptic strength is modified by long-term potentation (LTP) and long term depression (LTD) (Malenka and Bear, 2004). In animal models of depression it has been found that chronic, mild stress can impair hippocampal LTP (Alfarez *et al.*, 2003) and enhance LTD (Holderbach *et al.*, 2007). Furthermore, changes in a process, which includes cell proliferation, differentiation and migration (neurogenesis), are linked to the pathophysiology of MDD (Kempermann and Kronenberg, 2003, Nissen *et al.*, 2010). Animal models of depression showed decreased neurogenesis (Alonso *et al.*, 2004, Holderbach *et al.*, 2007) and an increase in newborn neurons upon treatment with antidepressants (Malberg *et al.*, 2000).

At the structural level, synaptic plasticity can occur either at the pre or the post synapse (Figure 5). The genes and proteins, which are involved in the cellular mechanism of plasticity, are many. The present study focused on four candidate genes, which were shown to be involved in the etiology of depression (Figure 5; Table 6).

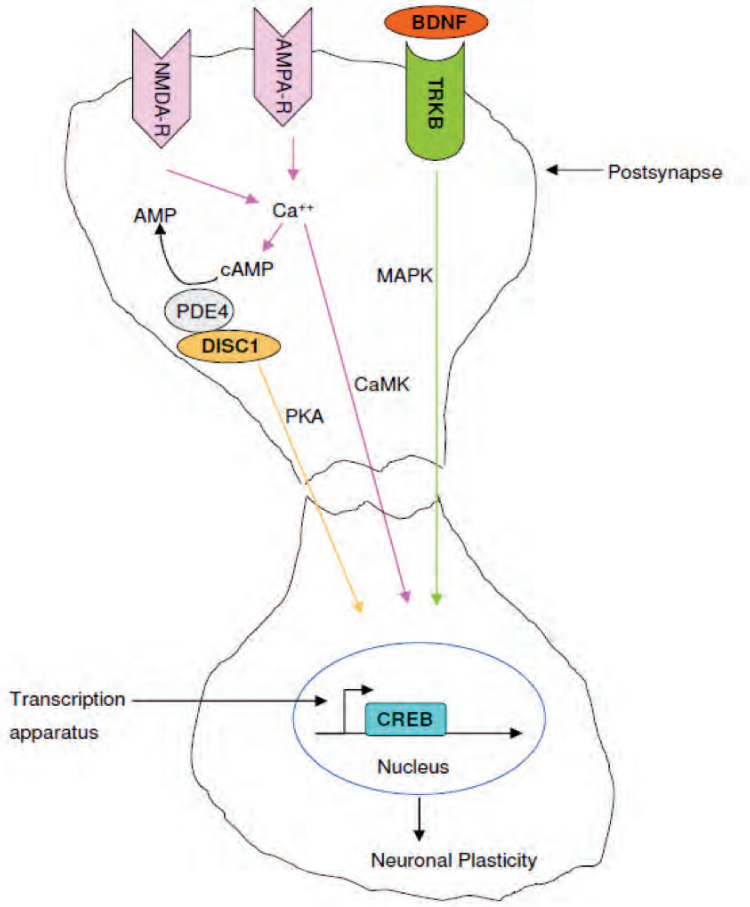


Figure 5.Schematic overview of the molecular pathways involved in neural plasticity [Figure modified from (Pittenger and Duman, 2008)]. Neuroplasticity is triggered by conductance of calcium ions (Ca^{++}) into the synaptic terminal. The local elevations in Ca^{++} increases cAMP postsynaptically, which leads to an activation of protein kinase A (PKA) and calcium-calmodulin-dependent kinase (CaMK) signaling. A mitogen-activated protein kinase (MAPK) signal transduction pathway is also involved in the plasticity. The MAPK cascade is triggered by an activation of neurotrophic tyrosine kinase, receptor (TRKB), on the binding of brain-derived neurotrophic factor (BDNF). Upon sufficient elevation of PKA, CaMK, and MAPK, signal transduction cascades transmit signals to the nucleus where the transcription factor, cAMP responsive element binding protein (CREB), which is involved in the regulation of synaptic change, is activated. These pathways are influenced by chronic stress and depression, have been associated with reductions in the transcription factor CREB (Pittenger and Duman, 2008). The cAMP–CREB cascade is up-regulated by antidepressant treatment (Manji et al., 2001, Castren, 2005). NMDA-R=N-methyl-D-aspartate glutamate receptor, AMPA-R=amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid glutamate receptor, PDE4=phosphodiesterase 4, DISC1=disrupted in schizophrenia 1.

Table 6. Candidate genes involved in the regulation of neuroplasticity.

Gene	*Name*	*Function*	*Chromosome*	*Reference in depression*
DISC-1	Disrupted in schizophrenia-1	Neurodeve-lopment and neural signaling	1q42	(Hashimoto *et al.*, 2006)
BDNF	Brain-derived neurotrophic factor	Plasticity	11p13	(Sen *et al.*, 2003, Schumacher *et al.*, 2005)
NTRK2 (TRKB)	Neurotrophic tyrosine kinase, receptor, type 2	Receptor for BDNF	9q22.1	(Dong *et al.*, 2009)
CREB1	cAMP responsi-ve element bin-ding protein 1	Transcription factor	2q33.3	(Zubenko *et al.*, 2003, Perlis *et al.*, 2007, Dong *et al.*, 2009)

The schizophrenia-1 gene (*DISC-1*) is located on chromosome 1q42 and spans a 415 kb region. It is highly expressed in the hippocampus and the cerebral cortex (Schurov *et al.*, 2004). Functionally, *DISC-1* is involved in neurodevelopment and synaptic modulation (Schurov *et al.*, 2004). *DISC-1* has been implicated in the regulation of cAMP signaling through interaction with PDE4, and it coordinates PKA-mediated signaling, which has a role in cell migration (Pawson and Scott, 2010). The carriers of the Cys704 allele of a genetic variant (Ser704Cys, rs821616) of the *DISC-1* gene, showed reduced gray matter volumes, linked to lower extracellular signal-regulated kinase activity, which has been suggested to have a role in the pathophysiology of MDD (Hashimoto *et al.*, 2006). Mutations in *DISC-1* are also associated with SCZ and BP (Hennah *et al.*, 2003, Palo *et al.*, 2007).

Brain-derived neurotrophic factor (BDNF) is a protein that belongs to the neurotrophin (nerve growth factor-related proteins) superfamily. The BDNF protein is coded by the *BDNF* gene, spanning a 67 kb region on chromosome 11p13. The physiological role of BDNF in the developing and adult CNS is to promote, modify, develop, and support the survival of neurons (Castren and Rantamaki, 2010). BDNF signaling is involved in neurogenesis, neuronal plasticity, and also in the pathophysiology in MDD (Castren, 2005). The serum level of BDNF is reduced in depressed patients, while antidepressant treatment can normalize the level (Castren and Rantamaki, 2010).

The neurotrophic tyrosine kinase receptor type 2 (NTRK2), also called TRKB, is a tyrosine protein kinase, which is encoded by the *NTRK2* gene. It is located on chromosome 9q22.1, spanning a 350 kb region. It mediates the plasticity-enhancing effects of BDNF (Castren and Rantamaki, 2010). *Postmortem* brain studies have revealed a reduction in hippocampal TRKB and BDNF mRNA levels in suicide victims with depression (Kohli *et al.*, 2010).

The *CREB1* gene encodes the cyclic AMP responsive element binding protein 1, located on chromosome 2q33.3, spanning a 69 kb region. CREB functions as a transcription factor and plays an important role in the regulation of cellular responses involved in the development and survival of neuronal cells, and in neuronal differentiation and memory formation (Lonze and Ginty, 2002). Increase in the level of calcium or cAMP in a synapse triggers the phosphorylation and activation of CREB. The *CREB* gene codes for three alternatively spliced activators α, β, and Δ (Silva *et al.*, 1998). CREB α, β, mutant mice demonstrate a significant increase in hippocampal neurogenesis, compared to wild-type mice, and they respond to acute antidepressant treatment (Gur *et al.*, 2007). In humans, sequence variations in the promoter and in intron 8 of *CREB1* have been found to be associated with mood disorders (Zubenko *et al.*, 2003).

HPA-axis

The hypothalamic-pituitary-adrenal axis (HPA-axis) is a complex functional system related to the regulation of stress and involving interaction between the hypothalamus, the pituitary gland and the adrenal gland. The HPA-axis plays an important role in the regulation of stress, sexuality, mood, sleep, and energy or fatigue (Hafez and Hafez, 2004, Buckley and Schatzberg, 2005, Gottschalk *et al.*, 2005, Holsboer and Ising, 2010). A stress response, triggers the release of corticotropin-releasing factor/hormone (CRF/CRH) from the hypothalamus, which activates CRH receptor 1 (CRHR1) in the pituitary gland, leading to the secretion of adrenocorticotropic hormone (ACTH) to the circulation. ACTH then travels to the adrenal glands, where it activates the secretion of glucocorticoids (cortisol, a stress hormone, in humans, and corticosterone in rodents). Cortisol then binds to corticosteroid receptors in the hypothalamus and the anterior pituitary, to inhibit further synthesis and release of CRF from the hypothalamus in a negative-feedback loop (de Kloet *et al.*, 2005). Dysfunction of corticosteroid receptors (glucocorticoid receptor (GR)) may lead to hyperactivity of the HPA-axis (Buckley and Schatzberg, 2005). HPA-axis hyperactivity is associated with sleep disturbances (Buckley and Schatzberg, 2005). An elevated cortisol level also associates with stress related disorders (Feder *et al.*, 2009), major depression (Keller *et al.*, 2006), as well as with sleep disturbances

associated depression (Arborelius *et al.*, 1999). Of the genes from the HPA-axis, *NR3C1* and the *CRHR1* were focused on here (Table 7), as they have been shown previously to be involved in depression.

Table 7. Candidate genes involved in the regulation of HPA-axis.

Gene	*Name*	*Function*	*Chromosome*	*Reference in depression*
NR3C1	Nuclear receptor subfamily 3, group C, member 1	Glucocorticoid receptor	5q31.3	(van West *et al.*, 2006)
CRHR1	Corticotropin-releasing hormone receptor 1	Corticotropin-releasing factor/hormone (CRF/CRH) receptor	17q12-q22	(Liu *et al.*, 2006, Bradley *et al.*, 2008, Wasserman *et al.*, 2009)

The glucocorticoid receptor (GR) is encoded by the nuclear receptor subfamily 3, group C, member 1 gene (*NR3C1*), which is located on chromosome 5q31.3 spanning a 157.58 kb region. It plays an important role in maintaining homeostasis after stress and in regulating nervous and immune system functions (Jokinen *et al.*, 2007). In the brain, GR modulates cognition, addiction, and emotional behavior (Tronche *et al.*, 1999). Various studies have suggested an impaired function, or decreased expression, of GR in patients with depression, for example, *postmortem* studies have revealed reduced GR levels in the hippocampus and in several other brain areas of patients with depression (Lopez *et al.*, 1998, Webster *et al.*, 2002).

The corticotropin-releasing-hormone receptor 1 gene (*CRHR1*), on chromosome 17q21-22 spanning a 20 kb region, encodes a G-protein coupled receptor. CRHR1 is expressed in the pituitary gland, where it mediates the action of CRF (Liu *et al.*, 2006). Genetic variants of the *CRHR1* gene are associated with depression (Liu *et al.*, 2006, Bradley *et al.*, 2008, Wasserman *et al.*, 2009).

2.1.4 Genetic background

***Heritability of depression*.** Heritability is defined as 'the proportion of phenotypic variance attributable to genetic variance' (Falconer, 1989). Heritability estimates for MDD range from 17% to 78% (Sullivan *et al.*, 2000). Both genetic and environmental risk factors are involved in the etiology of the disease (Kendler *et al.*, 1995, Caspi *et al.*, 2003, Farmer *et al.*, 2005). Stressful life events and traumatic childhood

events, such as abuse, often precipitate an onset of MDD (Williamson *et al.*, 1998, Caspi *et al.*, 2003). Genetic variations also modify the susceptibility to stressful life experiences and thereby the onset of MDD (Kendler *et al.*, 1995, Caspi *et al.*, 2003). A candidate gene study on a functional polymorphism (*5-HTTLPR*) in the upstream regulatory region of the serotonin transporter gene (*SLC6A4*) revealed an association with the onset of depression in the presence of life stress (Caspi *et al.*, 2003). The homozygote carriers of the S allele (SS) of *5-HTTLPR* showed more depressive symptoms, MDD diagnoses, and suicidality in relation to stressful life events than those homozygous for the L allele (LL) ($P<0.001$) (Caspi *et al.*, 2003). A recent meta-analysis on studies published up to 2006 also reported a significant association between the *5-HTTLPR* alleles (S vs. L) and MDD (Odds ratio (OR) for S allele=1.11) (Lopez-Leon *et al.*, 2008). However, this report did not include any information on gene-environment (GxE) interaction (Lopez-Leon *et al.*, 2008). Another meta-analysis study of GxE interaction found that *5-HTTLPR* genotypes (SS, SL, or LL), alone or in interaction with stressful life experiences, were not associated with increased risk of depression, either in men or women (Risch *et al.*, 2009). Furthermore, a meta-analysis exploring the GxE interaction, up to 2009, supports the hypothesis that *5-HTTLPR* moderates the relationship between stress and depression (Karg *et al.*, 2011). Therefore, some authors suggest that *5-HTTLPR* may not directly associate with MDD but modulate the effect of serotonin on stress (Uher and McGuffin, 2008). However, the discrepant findings of GxE may originate from differences in methodology, definitions of the phenotype, or the quality of environmental exposure measurements (Caspi *et al.*, 2010).

Gender. Depressive disorder is more prevalent in women than in men (Kessler *et al.*, 1993, Bebbington, 1996, Kuehner, 2003) which raises the possibility that some gender-related factors could play a role in MDD. The ratio of depression prevalence female/male is 2:1 (Jennet, 1977). The heritability estimate for MDD in women was 42%, and in men 29%, according to a twin study (Kendler *et al.*, 2006). A twin study showed that females without a history of depression and who had experienced stressful life events, such as divorce or a death of a loved one, had an increased risk for developing depression (by 6%) (Kendler *et al.*, 2000). On the other hand, those with a history of family depression were 14% more likely to develop depression (Kendler *et al.*, 2000). The higher rate of depression in women may be due to stresses from adolescence emotions, and reproductive events (Ruderman and O'Campo, 1999). Similarly, a study of female mice has shown that the expression of serotonin transporter gene was influenced through transcriptional modification by sex hormones (Gubbels Bupp *et al.*, 2008). During stressful events, there is a lower rate of serotonin synthesis in females making them more susceptible to MDD than men (Nishizawa *et al.*, 1997). This may be further compounded in both women and men by a functional polymorphism (*5-HTTLPR*) in the serotonin transporter gene (*SLC6A4*) that can also modulate the effect of serotonin on stress (Kendler *et al.*,

1995, Caspi *et al.*, 2003). Moreover, corticotropin-releasing factor (CRF), a mediator of the stress response, is dysregulated in stress-related psychiatric disorders (Bangasser *et al.*, 2010). During stress, CRF activates the locus ceruleus (LC), the source of the brain norepinephrine system that regulates emotional arousal (Bangasser *et al.*, 2010). There is evidence for estrogen regulation of CRF gene expression, interaction with neurotransmitter systems that can induce sex-specific changes in mood (Dzaja *et al.*, 2005, Bangasser *et al.*, 2010). Hence, gender differences between females and males are most likely a combination of biological, environmental, and psychological factors.

2.2 Sleep

Sleep is a behavioral state characterized by reclined position, closed eyes, decreased movement, and decreased responsiveness to the internal and external environment (Markov and Goldman, 2006), as well as specific timing in the day as part of the circadian sleep-wake activity cycle (Zee and Vitiello, 2009).

Sleep disturbances. Disturbed sleep is characterized by difficulties to fall asleep, waking up during the night, early morning awakenings, waking up feeling unrefreshed, fatigue (tiredness, feeling of weariness, or lack of energy), and day time sleepiness (Grandner and Kripke, 2004). The disturbed sleep observed in depressed patients usually includes decreased sleep efficiency (a ratio of amount of time spent in bed/total sleep time) and sleep duration (Kupfer and Reynolds, 1992). One polysomnographic study showed 85% sleep efficacy in healthy controls and 72% in depressed patients (Leistedt *et al.*, 2009). Depressed patients also suffer from decreased slow wave sleep (SWS), increased REM sleep, and disturbances in sleep continuity (Srinivasan *et al.*, 2009). Disturbed sleep patterns, like short or prolonged sleep duration and insomnia, are associated with an increased risk to mental and physical health. To sleep less than 6 hours per night is a risk factor for acute myocardial infarction, hypertension, and coronary heart disease (Liu and Tanaka, 2002, Ayas *et al.*, 2003, Gangwisch *et al.*, 2006). Short (<6 hours per night) and long sleep (>8 hours per night) can both predispose to developing diabetes (Chaput *et al.*, 2009) and also depression (Perlman *et al.*, 2006). Sleep disturbances in depression have been suggested to originate from disturbances in the neurotransmitter system, as well as disturbances in the circadian system, which is also involved in the regulation of sleep (Armitage, 2007).

2.2.1 Regulation of sleep

According to a widely accepted sleep regulation model, there are two biological factors which regulate sleep. Factor 1 is a sleep-wake dependent homeostatic component (Process S), and factor 2 is a sleep-wake independent circadian component (Process C). The interaction of these two factors is described by the two process sleep regulation model (Borbely, 1982) (Figure 6).

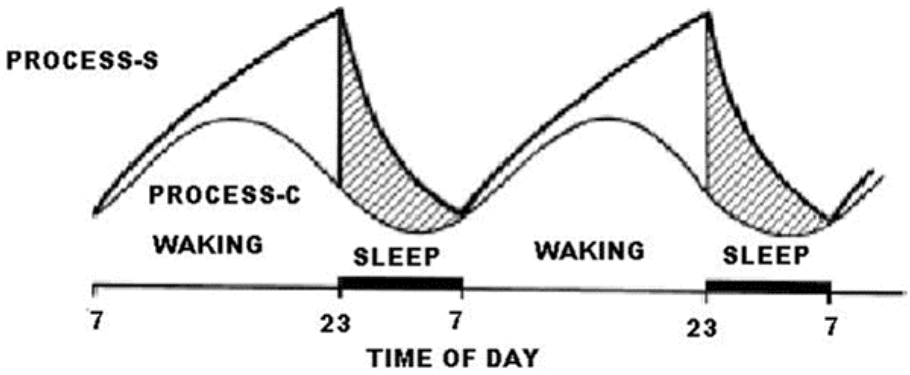


Figure 6. The two process model of sleep regulation [Figure modified from (Borbely and Achermann, 1999)]. The graphical representation of the upper curve displays the homeostatic (Process-S) and the lower curve the circadian rhythm cycle (Process-C), the x-axis indicates time of day, the dark black bar indicates the times where sleep normally occurs. Process-S indicates sleep propensity, the accumulated sleep pressure which increases during waking and declines during sleep. Sleep occurs when Process-S is at its peak and Process-C is declining.

2.2.1.1 Sleep homeostasis (Process-S)

The homeostatic regulation of sleep can be explained as a consequence of the accumulation of some substance during waking. Process-S is controlled by various interacting neural systems in the brain such as the hypothalamus, basal forebrain (BF), and brainstem nuclei (Borbely, 1982, Espana and Scammell, 2004). It has been suggested that wakefulness, through consumption of brain energy resources, results in a depletion of brain energy, more specifically a depletion of brain glycogen stores (Benington and Heller, 1995). Adenosine is the end-product of the break-down of ATP, being a signal of energy shortage. The extracellular levels of adenosine increase during prolonged wakefulness in the BF of both cats and rats (Porkka-Heiskanen *et al.*, 1997, Basheer *et al.*, 1999). Adenosine promotes sleep through adenosine A1 (A1R) or A2A receptors. Injection of an A1R receptor agonist into the BF area in rats (Basheer *et al.*, 1999), or of A2A receptor agonist into the subarach-

noidal space, increases sleep (Satoh *et al.*, 1998). It has been proposed that the increase in extracellular adenosine concentration during waking could inhibit the activity of cholinergic cells, which act as wakefulness promoting cell groups in the BF (Porkka-Heiskanen, 1999). Inhibition of the wake-promoting cells induces sleep, and during sleep the extracellular adenosine concentrations decrease and waking becomes possible again (Porkka-Heiskanen, 1999).

2.2.1.2 Circadian system (Process-C)

The circadian rhythm consists of the rhythmic activity of various physiological processes over a period of approximately 24 hours. It controls many biological and physiological processes in the body, including the sleep-wake cycle, digestive and hormonal activity, cell proliferation, and body temperature (Stratmann and Schibler, 2006). The master biological clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Hastings, 1998). The SCN synchronizes the circadian oscillators of neuronal cells, as well as peripheral cells, as far as they are not light sensitive (Schibler *et al.*, 2003). If the SCN is lesioned, the rhythms become desynchronized and can function with different periods (Edgar *et al.*, 1993). The SCN is situated above the optic chiasm (Ibata *et al.*, 1999, Abrahamson and Moore, 2001). Light stimulation to the SCN is mediated through the retino-hypothalamic tract (RHT), which originates from retinal ganglion cells which contain the light sensitive photopigment molecule melanopsin (Berson *et al.*, 2002). In addition to light signals, the SCN also receives nonphotic input via serotonergic projections from the midbrain raphe nuclei and also from the retinorecipient subcortical nuclei such as the ventral lateral geniculate nucleus (vLGN) and the intergeniculate leaflet (IGL) (Harrington, 1997, Turek, 1998). Furthermore, the light signal passes on through the SCN to the pineal gland, which synthesizes and releases melatonin (Moore and Lenn, 1972, Richardson, 2005). Melatonin secretion increases in darkness while light evokes an immediate decrease (Ueyama *et al.*, 1999). The intrinsic circadian period in humans is slightly longer than 24 hours (Wulff *et al.*, 2009). Therefore, the circadian system needs a daily synchronization (entrainment) of the endogenous oscillator to the solar day (Wulff *et al.*, 2009). The activation of SCN neurons by direct neural input from the retina via the RHT adjusts the biological clock to work in phase with the 24 h light-dark cycle (Moore and Lenn, 1972). The circadian system provides wake stimulus and counteracts the accumulating homeostatic sleep pressure during waking hours (Edgar *et al.*, 1993).

Overall, in the ‘regulation of sleep’, Process-C determines the timing of sleep whereas Process-S maintains the intensity and duration of sleep (Wulff *et al.*, 2009).

2.2.1.3 Genetic regulation

Circadian genes behind the regulation of sleep and depression. Genes involved in the generation of circadian rhythms (~24-hour cycles) are called clock genes. The light-dark cycle stimulates molecular events in the circadian clock (Bunney and Bunney, 2000). Various biological clock components act as transcriptional activators or repressors, generating feedback loops of the core circadian genes which maintain endogenous clock rhythms. The aryl hydrocarbon receptor nuclear translocator-like protein (*ARNTL*, alias *BMAL1*) and the clock homolog protein (*CLOCK*, alias *bHLHe8*) genes are the core components (Figure 7).

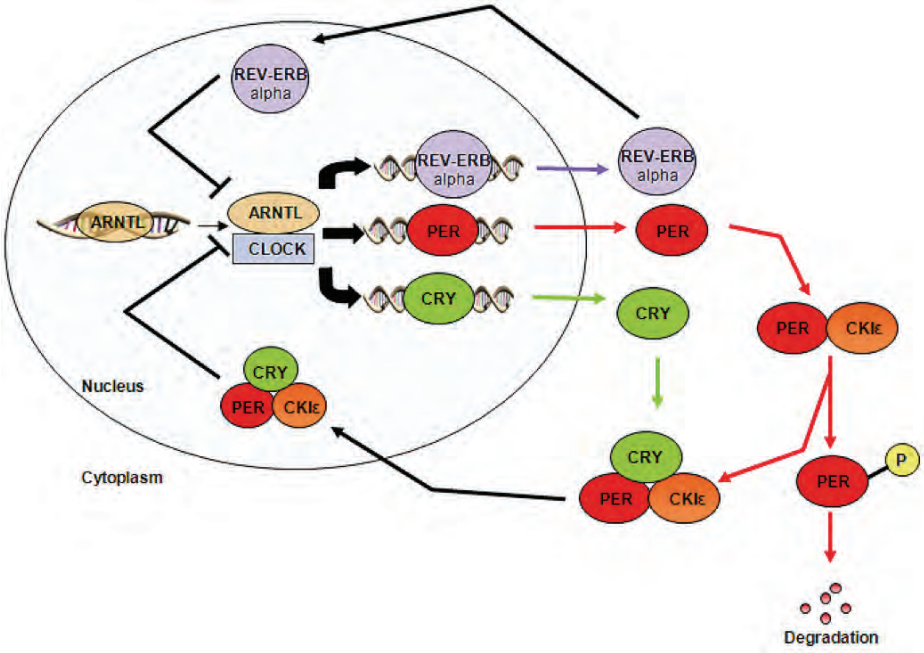


Figure 7. Schematic presentation of clock gene transcriptional/translational feedback loops in the SCN of mammals [Figure modified from (Fu and Lee, 2003)]. At the begning of the circadian day, in response to light, *ARNTL* expression levels are high whereas *CLOCK* remains steady (Fu and Lee, 2003). The high activity level of *ARNTL* in the nucleus promotes the formation of a heterodimer complex of *ARNTL* with either *CLOCK* or *NPAS2* (alias *bHLHe9*) (not shown in figure). Once formed, this complex binds to E-box elements in the promoter of three period genes (*PER1*, *PER2* and *PER3*) and two cryptochrome genes (*CRY1* and *CRY2*), as well as to other feedback loops including the transcription factors orphan nuclear receptors, *REV-ERBα* and *RORα* gene (alias *RORA*), thereby activating their transcription (Hastings *et al.*, 2003). After transcription and translation, negative feedback is achieved by the PER and CRY heterodimer complex, REV-ERBα, and RORα (Not shown in figure) proteins, which translocate back to the nucleus from the cytoplasm to suppress their own expression by inhibiting the ARNTL and CLOCK complex (Kume *et al.*, 1999). Late in the subjective day, a

post-translational modification takes place for the accumulated PER and CRY proteins in the cytoplasm (Hastings *et al.*, 2003). The casein kinase 1, epsilon (*CKIε*) phosphorylates PER proteins whereas unstable forms are degraded. The CRY proteins promote the formation of stable PER/ CKIε /CRY complexes that enter the nucleus during night time, where they inhibit the *ARNTL*, *PER*, *CRY* and *REV-ERBα* transcription (Preitner *et al.*, 2002). The interacting feedback loop ensures that at the beginning of a new circadian day, the levels of PER and CRY are low (Fu and Lee, 2003). A number of other core clock genes (not shown in figure) (Table 13), such *BHLHE41* (alias *DEC2*), *TIMELESS*, *TIPIN*, *NFIL3*, *BHLHE40*, *NR1D1*, *DBP*, are directly or indirectly participating in the work of molecular clock. *ARNTL*=aryl hydrocarbon receptor nuclear translocator-like protein, *CLOCK*=clock homolog protein, *NPAS2*=neuronal PAS domain protein 2, *PER1*=Period homolog 1 (Drosophila), *PER2*=Period homolog 2 (Drosophila), *PER3*=Period homolog 3 (Drosophila), *CRY1*=Cryptochrome 1 (photolyase-like), *CRY2*=Cryptochrome 2 (photolyase-like), *REV-ERBα*=Nuclear receptor subfamily 1, group D, member 1, *RORA*=RAR-related orphan receptor A, *CSNK1E* (alias *CKIε*)=casein kinases-1 epsilon, *BHLHE41*=basic helix-loop-helix family, member e41, *TIMELESS*=timeless homolog (Drosophila), *TIPIN*=TIMELESS interacting protein, *NFIL3*=nuclear factor, interleukin 3 regulated, *BHLHE40*=basic helix-loop-helix family, member e40, *NR1D1*=nuclear receptor subfamily1, group D, member1, *DBP*=D site of albumin promoter (albumin D-box) binding protein.

A disruption of circadian oscillators can trigger sleep abnormalities and mood disorders in humans. For instance, in familial advanced sleep phase syndrome (FASPS), a missense mutation within the *CKIε* binding region of *PER2* causes hypophosphorylation of the PER2 protein (Toh *et al.*, 2001, Xu *et al.*, 2005). Polymorphisms in *PER1* and *PER3* are associated with the diurnal preference and delayed sleep phase syndrome (DSPS) (Ebisawa *et al.*, 2001, Archer *et al.*, 2003, Carpen *et al.*, 2005). A mutation in a transcriptional factor, *BHLHE41* (*DEC2*) gene has been associated with short sleep, both in humans and mice (He *et al.*, 2009). Furthermore, the 3111T/C polymorphism of *CLOCK* was proposed to have a role in sleep disregulations in mood disorders (Serretti *et al.*, 2003). MDD patients with the C allele were reported to be more prone to suffer from insomnia while BP patients with the same allele showed insomnia throughout the whole night and a reduced need for sleep (Serretti *et al.*, 2003). In addition, in another study, this *CLOCK* polymorphism was associated to the time course of insomnia during antidepressant treatments (Serretti *et al.*, 2005). Other circadian genes such as *NPAS2*, *CRY2*, and *PER2* polymorphisms have also been reported to associate with MDD (Soria *et al.*, 2010, Lavebratt *et al.*, 2010). In addition, circadian clock-related polymorphisms were involved in susceptibility to seasonal affective disorder (SAD) and diurnal preference (morningness-eveningness) tendencies (Johansson *et al.*, 2003). The individuals with a behavioural preference for "eveningness" have a greater tendency to develop depression (Barnard and Nolan, 2008).

Heritability of sleep. A study on 2238 monozygotic (MZ) and 4545 dizygotic (DZ) adult Finnish twins demonstrated that the self-reported sleep duration and sleep

quality have heritability estimate, 44% in both (Partinen *et al.*, 1983), whereas the heritability for sleep quality in Australian twin pairs was 33% (Heath *et al.*, 1990). Moreover, twin studies have demonstrated that human EEG heritability during waking was 81% (van Beijsterveldt and van Baal, 2002), while heritability of EEG during NREM sleep was even 96% (De Gennaro *et al.*, 2008).

2.2.2 Epidemiology of sleep

An epidemiological study of self-reported sleep duration suggests that 18-25% of the population suffer from insufficient or poor sleep (Kronholm *et al.*, 2006). A Finnish twin study suggests that sleeping less than 7 hours per night increases an incidence of depressed mood (Paunio *et al.*, 2009). Several epidemiological studies suggest that sleeping from 7 to 8 hours per night is considered optimal for health, since self-reported sleep durations of less than 7 hours or more than 8 hours are linked with increased morbidity and mortality (Hublin *et al.*, 2007, Wallander *et al.*, 2007, Kronholm *et al.*, 2011). According to a cross-sectional population-based study conducted in the United States, 28.3% reported sleep durations of 6 hours or less, while 8.5% reported sleep durations of 9 hours or more (Krueger and Friedman, 2009). The prevalence of short sleepers ($\leq$ 6 hours) in the Finnish adult population was estimated to be 14.5% (16.7% of men and 12.5% of women) and that of long sleepers ($\geq$ 9 hours) 13.5% (10.5% of men and 16.1% of women) (Kronholm *et al.*, 2006). Studies based on sleep diary data and self-reported sleep duration have also shown that women overall sleep more than men, while their sleep quality is lower compared to that of men (Krueger and Friedman, 2009, Chatzitheochari and Arber, 2009). Insomnia is also more prevalent in women (14.9%) than in men (7.4%) (Hublin and Partinen, 2002, Ohayon and Partinen, 2002). The gender differences in sleep duration or poor quality of sleep may arise due to factors like sociological, physiological, and hormonal characteristics (Dzaja *et al.*, 2005, Arber *et al.*, 2009).

2.2.3 Etiology and pathophysiology

Functional regulation of sleep is essential for a healthy life, and genetic factors are likely to contribute to a susceptibility to develop disturbed sleep (see in section 2.2.1.3). Several neurotransmitter pathways are involved in the regulation of the sleep-wake cycle. Monoaminergic neurons are active during wakefulness, will slow down during NREM sleep, and remain inactive during REM sleep (Saper *et al.*, 2005). Destruction of serotonergic neurons of the raphe nuclei leads to severe insomnia (Jouvet, 1999). There is also evidence that catecholamines play a role in sleep and waking (Irwin *et al.*, 1999). The dopaminergic system plays important role

in behavioral wakefulness (Monti and Monti, 2007). An increase in the extracellular level of dopamine metabolites (DOPAC and HVA) is seen in the BF during sleep deprivation (SD), indicating maximum dopamine release during waking (Zant *et al.*, 2011). The HPA-axis hyperactivity, with an increase in ACTH and cortisol secretion, and CRF response to stress (see in section 2.1.3, HPA-axis) is associated with sleep disturbances: sleep fragmentation, night-time awakenings or shortened sleep time, and decreased slow-wave sleep (Buckley and Schatzberg, 2005, Hudson and Bush, 2010). GABAergic cells in the anterior hypothalamus and basal forebrain are most active during sleep (Gritti *et al.*, 1994), and lesions along this pathway (containing hypothalamic area, basal forebrain, and cerebral cortex) produce sleepiness or coma (Saper *et al.*, 2005).

2.3 Sleep and mood

Disturbed sleep is one of the symptoms of depressive disorder. It diminishes daily happiness and produces depressed mood. However, sleep deprivation or sleep loss have mood elevating effects on patients with depression (Wu and Bunney, 1990, Adrien, 2002), in whom lower mood was reduced by one night of sleep deprivation, while depressive symptoms reoccurred after one night of recovery sleep (Tsuno *et al.*, 2005). Among the different phases of sleep, REM sleep is mainly involved in the modulation of daily mood. REM sleep pressure is increased in patients with depression (Vogel *et al.*, 1980). Selective REM sleep deprivation with the according nocturnal awakenings over a period of three weeks has shown to induce an antidepressant effect on the mood of depressed subjects (Vogel *et al.*, 1980), while selective deprivation of NREM sleep did not lead to clinical improvement (Vogel *et al.*, 1980). Several studies have tried to elucidate the mechanisms of action of sleep deprivation. There is a persistent reduction in glucose metabolism in the anterior cingulate cortex of patients with depression (Smith *et al.*, 1999). According to a PET study, glucose metabolism in the cingulate cortex is elevated among those depressed patients who improved after sleep deprivation (responders), but not in the nonresponders (Riemann *et al.*, 2001). MDD is associated with a decrease in serotonergic neurotransmission and increased cholinergic tone which leads to an enhanced REM sleep pressure (Adrien, 2002). Sleep deprivation appears to influence also the activity of brain serotonin transmission, and prolonged wakefulness is associated with release and increased extracellular concentrations of 5-HT (Zant *et al.*, 2011), and down-regulation of 5-HT_{1A} autoreceptors (Portas *et al.*, 2000). Genetic studies of serotonin transporter (*SLC6A4, 5-HTTLPR*) suggest that patients with depression, homozygous for the L allele, are better responders to sleep deprivation compared to patients with the S allele (Benedetti *et al.*, 1999). Thus, the effects of sleep deprivation in depressed patients may induce enhancement of serotoninergic neurotransmission,

which alleviates REM sleep pressure and leads to significant mood improvement. All of these findings suggest that the regulation of sleep and mood are interlinked.

2.4 Studying the human genome

2.4.1 The Human Genome Project (HGP) and the Human Haplotype Map Project (HapMap)

Genes are made of deoxyribonucleic acid (DNA) and are found on chromosomes in the nucleus of the cell. The genetic instructions contained in a genome are encoded in DNA, which has a long sequence of four chemical bases abbreviated as A (adenine), C (cytosine), G (guanine), and T (thymine), called nucleotides (Watson and Crick, 1953). At the beginning of the 21st century, two landmarks in the history of biomedical sciences were achieved, the Human Genome Project (HGP) (www.ornl.gov/hgmis) and the Human Haplotype Map Project (HapMap) (http://hapmap.ncbi.nlm.nih.gov/). The HGP was an international research effort to sequence the entire human genome in order of nucleotides along the DNA molecule in each chromosome (The International Human Genome Sequencing Consortium, 2004). The sequencing resulted in the current estimate of the number of genes to be 25,000 and the number of protein coding genes to be over 18,000. The average gene consists of approximately 3000 bases (Venter *et al.*, 2001, Lander *et al.*, 2001). In the human genome sequence, 99.9% of the bases are identical in all humans, while the remaining 0.1% variation makes every individual unique. Table 8 summarizes several forms of variations present in the human genome. These variations range from single nucleotides to gross alterations in the DNA sequence. Genetic variations may have ability to interfere with gene expression and their protein function (Eichler *et al.*, 2007). These variations might contribute to new or heritable diseases or have an impact on human phenotype (Eichler *et al.*, 2007).

Table 8. Types of polymorphisms in the genome [Adapted from (Tabor *et al.*, 2002, Feuk *et al.*, 2006, Sharp *et al.*, 2006, Richard *et al.*, 2008, The 1000 Genomes Project Consortium, 2011)].

Type of variant	*Functional effect*
1) SNPs	
A) Coding SNPs	
– Synonymous/ Sense/Silent mutation	Create a new codon that still codes for the same amino acids, does not change the amino acid in the protein, but can alter splicing
– Non-synonymous/ Missense /Point mutation	Create a new codon that codes for new amino acids, changes an amino acid in protein to one with similar properties
– Nonsense/Stop-introducing SNPs	Change an amino acid to stop codon results in a premature termination of amino acid sequence
B) Noncoding SNPs	
– Promoter/Regulatory (5′ UTR, 3′ UTR)	Does not change the amino acid but can affect the location, timing or level of gene expression
– Intergenic	SNP could affect transcription factor binding sites, enhancer regions, and influence gene transcription
– Intronic	No known function, but might affect expression or mRNA stability
– Splice site/Intron–exon boundary	Might change the splicing pattern or efficiency of introns
2) Insertions/Deletions/Duplication	
– Frameshift	Insertion or deletion of one base in the triplet reading frame, changes the frame of the protein-coding region
– Variable number of tandem repeats (VNTRS)	
• Microsatellite	Short repeats of one to five base pair units, or varying from 10 bases up to 500 bases. It might influence the regulation of gene expression
• Minisatellites	Consist of tandem repeats spanning approximately 100 bp to 20 kb in length, valuable tools for genetic analysis
– Copy number variants (CNVs)	A segment of DNA that appear in 1kb or larger size in different numbers of copies in different people
– Low copy repeat/Segmental duplication	A segment of DNA >1 kb in size with >90% sequence identity which occurs in two or more copies per haploid genome

Single nucleotide polymorphisms (SNPs) are the most researched variations in the human genome. These are DNA variations which replace one of the four nucleotides for another. In the human genome, SNPs can be found on average in 1 out of every 300-1000 nucleotides, and are much more common within genes (The International

HapMap Consortium, 2003). SNPs are presented most typically as bi-allelic markers, out of which the allele with the lower frequency is called the minor allele and one with the higher frequency is referred to the major allele. In the human population, there are an estimated 11 million SNPs that have minor allele frequencies of 1% (Kruglyak and Nickerson, 2001). Currently there are over 52 million SNPs recorded on the NCBI dbSNP database (Build 135, released in 2011), of which 41 million are validated (http://www.ncbi.nlm.nih.gov/snp).

The International HapMap project was launched in the year 2002 with the aim of cataloging and mapping common patterns of human genetic variation (The International HapMap Consortium, 2003). The results of the HapMap project were published in three phases (The International HapMap Consortium, 2005, Frazer *et al.*, 2007, Altshuler *et al.*, 2010). Samples were genotyped from European, African, and Asian populations, resulting in an open-access public database for the scientific community (http://hapmap.ncbi.nlm.nih.gov/). The HapMap project discovered a block-like structure in human genome (Figure 8) with regions of limited diversity. The project catalogued allele frequencies in different global populations, identified 'tagging SNPs' (SNPs that tag with haplotype blocks) and created correlation patterns between nearby variants (a phenomenon known as linkage disequilibrium (LD)) over 3.5 million SNPs, and produced a resource for LD-based marker selection for genetic studies (The International HapMap Consortium, 2005, Frazer *et al.*, 2007). For a deeper understanding of the role of inherited human DNA variations, a 1000 Genomes Project was initiated in 2008 (http://www.1000genomes.org) with the goal of increasing the catalog of genomic variations by using next-generation sequencing technologies (Durbin *et al.*, 2010). This project will provide information on rare variants in specific genes in large disease-or population-based cohorts, new methods for accurate imputation of variants in genome-wide association studies (GWAS), and offer a platform for studies using genome-wide sequence data (Durbin *et al.*, 2010).

2.4.2 Designs for molecular genetic studies

Identification of the underlying genetic risk factors in classical Mendelian monogenetic disorders is a relatively straightforward process. Studies on these disorders have also shown how single mutated disease genes can be both necessary and sufficient to cause the observed trait. In contrast, identification of genetic risk factors for complex diseases (as for example depression, bipolar disorder, diabetes, or cardiovascular disease) is far more challenging, as those disregard the single-gene dominant or single-gene recessive Mendelian law. Furthermore, etiology of complex disease is heterogeneous, involving both environmental and genetic risk factors which may act together or independently (Cardon and Bell, 2001). The yield of ge-

netic studies on complex traits has remained controversial, partially due to diagnostic uncertainties, to mode of inheritance, incomplete penetrance, multiple disease susceptibility loci, variable age of onset, epistasis, assortative mating, gene environment interaction, or proportion of phenocopies, study designs such as candidate gene studies, and small sample sizes (Nothen *et al.*, 1993). In designing a genetic dissection, some crucial choices related to sampling and phenotyping strategies, as well as to analytical methods, arise. These will be discussed in the following section.

Sampling

The sample size is a key determinant when the power of the study is considered. The power of a sample to detect significant association is measured by power calculations, and positive findings will ideally need to be replicated in bigger samples (Wacholder *et al.*, 2004). Careful sampling design is crucial to avoid sampling bias or error. The samples used in research should be representative of the population of interest. Various sampling strategies are commonly used in molecular genetic studies.

A *cross-sectional study* is conducted on the entire population or a subset thereof. In this type of design, data are collected at a defined time. This kind of sampling can provide estimates for disease prevalences. This type of sampling is not suitable for rare diseases due to the small number of affected individuals (Cordell and Clayton, 2005).

A *longitudinal cohort* design is often used for genetic association studies and is useful if the examined trait is relatively frequent in the population, or if it is quantitative by its nature. This study design follows the disease incidence over a long time and the participants are observed in order to identify new incident cases of the disease. This sampling can provide estimates for disease incidence, and collection of information from the environment can be achieved easily. However, the follow-up of participants is usually expensive and time consuming, and dropout of participants occurs during the follow-up. Longitudinal studies are also not ideal for rare diseases which require large numbers of study participants (Cardon and Bell, 2001).

A *case-control sample* strategy has been widely applied in etiological studies on complex diseases (Spinka *et al.*, 2005). In this strategy, cases and controls are ascertained from the same population. Case-control sampling is effective in the study of rare diseases. Issues such as family history, age of onset, or severity of disease are important to consider while selecting cases (Teng and Risch, 1999). In case-control samples, differences in the frequency of susceptibility alleles between cases and controls can be defined (Hattersley and McCarthy, 2005). The selection of the control sample is crucial, and issues such as health status, age, and gender must be taken

into account. The controls must be selected from the same population as the cases in order to avoid findings due to population stratification (Cordell and Clayton, 2005).

A *family-based sampling* strategy, a sample of parents and their affected and unaffected offspring, is a powerful method to identify genetic associations. In family-based association approaches, alleles transmitted to affected offspring are compared to the nontransmitted alleles (Lange *et al.*, 2008). These types of samples are efficient for the identification of rare, population-specific risk factors, and for the validation of previously identified genetic factors. The sample design is ideal for avoiding spurious results due to population stratification. Sample collection is, however, expensive.

Twin samples can be used to assess genetic and environmental factors, which produce differences in a trait of interest. If monozygotic twins exhibit higher concordance for a trait as compared to dizygotic, genetic factors are likely to play a role in the development of the trait (Kaprio and Koskenvuo, 2002). Twin studies provide estimates of trait heritability on the basis of the concordance rates in monozygotic and dizygotic twins. The disadvantage of twin sampling is that information about zygosity is often not available in medical records, and the sample size is small due to the difficulty of getting suitable sets of twins.

Phenotyping

Phenotypes are the observable characteristics of an organism to which both genetic and environmental factors contribute (Rice *et al.*, 2001). Phenotypes can be observed through signs, symptoms, visible traits, and they are often inherently dynamic and unstable (Schulze and McMahon, 2004). In genetic research, phenotypic traits are basically of two kinds, quantitative and qualitative. Certain traits such as blood pressure, weight, or performance scores in a neuropsychological test vary continuously across individuals and can be handled as quantitative traits. Qualitative phenotypes are usually dichotomized traits that can be described as binary data if there are only two categories (as for example cases and controls for a disease). A complex disease is genetically heterogeneous that complicates the detection of the disease contributing factors (Schork, 1997). Therefore, a common method for identifying phenotype is to rely on medically recognized criteria. For example, the diagnosis of MDD which depends on DSM criteria show clusters of symptoms and characteristics of clinical course that reliably differentiate depressed patients from normal individuals (Hasler *et al.*, 2004). Thus, diagnostic assessment of phenotypes requires a standard framework of clinical checklists or examination items, questionnaires, and laboratory data (Schulze and McMahon, 2004). Several studies have used this scheme for the identification of depressive phenotype. Caspi et al (2003) showed that 5-HTT genotypes moderate the influence of stressful life events on MDD (Caspi

et al., 2003). Thus increased stress sensitivity can be considered to present psychopathological endophenotype for MDD. Sleep disturbances, including insomnia, have been found to prominently contribute toward the risk of developing MDD (Breslau *et al.*, 1996). Furthermore, REM sleep abnormalities have been found in patients with MDD and they are proposed as biological endophenotypes for genetic studies (Hasler *et al.*, 2004). The term endophenotype is understood as any hereditary characteristic that is normally associated with disease but is not a direct symptom of that disease (Gottesman and Gould, 2003).

Analytical methods

Linkage analysis. This analytical method is typically carried out to determine chromosomal locations for disease susceptibility loci by genotyping genetic markers in *pedigrees with multiple affected individuals.* The method helps to reveal the chromosomal region that is transmitted with the disease. In linkage, loci located close together on same chromosome are not usually separated by a crossover event (recombination) but inherited together during meiosis. The genetic distance between two loci is measured by the recombination fraction [θ]. If two loci lie close together, θ is 0, while θ is 0.5 if these loci are independently segregating. The logarithm of odds (LOD) score is a likelihood ratio test used for calculating the likelihood that two loci co-segregate together because of linkage instead of chance (Morton, 1955). In genome-wide analyses, evidence of linkage to depression has been found for several genomic regions including 1p36, 12q23.3-q24.11, 13q31.1-q31.3 (McGuffin *et al.*, 2005), 15q25.3-26.2 (Holmans *et al.*, 2004), 3p12.3-q12.3, 18q21.33-q22.2 (Camp *et al.*, 2005), and 3p26-3p25 (Breen *et al.*, 2011).

Association analysis. In this method, the frequencies of alleles, genotypes or haplotypes of genetic markers are compared between groups of people with a certain disease to a control group of people without the disease. This method allows the detection of genetic effects in samples of various types, including case-control samples to population-based cohorts or family-based samples. A minor bias in sample ascertainment can produce errors in the estimates of allele frequencies. In addition, testing of multiple genetic markers tends to increase the risk for false positive results. Therefore, replication of results is usually required to confirm an association. To avoid population stratification effects, cases and controls have to be carefully matched regarding their geographical, social, and ethnic background (Nothen *et al.*, 1993). As compared to linkage analysis, association analysis has a greater statistical power to discover variants with relatively small effect (Risch and Merikangas, 1996). The effect size in association analyses is measured by the odds ratio (OR), defined as ‘the odds of exposure among cases divided by the odds of exposure among controls’ (Zondervan and Cardon, 2004). The value OR=1 means that exposed indi-

viduals are at no increased risk compared with the controls. OR>1 indicates an increased risk and OR<1 implies protective effect (Zondervan and Cardon, 2004).

An association study using a *candidate gene approach* is based on a prior hypothesis that a candidate gene has an etiological role in a disease. The selection of variants is based on the structure of the gene, in which linkage disequilibrium (LD) between markers in the region of a gene is taken into account. If variants are in complete LD, it is possible to deduct their genotypes on the basis of a single genotype (Figure 8). This strategy covers a relatively wide range of genetic variation in the region at a reasonable cost. LD can be observed in a population as block like structures, such as haplotype or LD blocks (The International HapMap Consortium, 2005). LD is a measure of two alleles segregating together (positive LD) or not. The most commonly used measures for LD, pair-wise method, which is denoted as the D prime measure of LD (D'), or as the squared correlation coefficient (r^2). The value of both D' and r^2 of 0 suggests that two markers have been separated by recombination. Furthermore, both D' and r^2 equal 1 suggests that the markers are in complete LD. Recombination events may break down LD in successive generations (Hill and Robertson, 1966). Therefore, a random marker within, or in close proximity to, a gene may not necessarily be in a complete LD with a disease-predisposing mutation in the same gene. In particular, reconstructing single nucleotide polymorphism haplotypes and testing those for an association to the disease should help in refining the chromosomal background on which the actual disease-predisposing mutation occurs (Daly *et al.*, 2001).

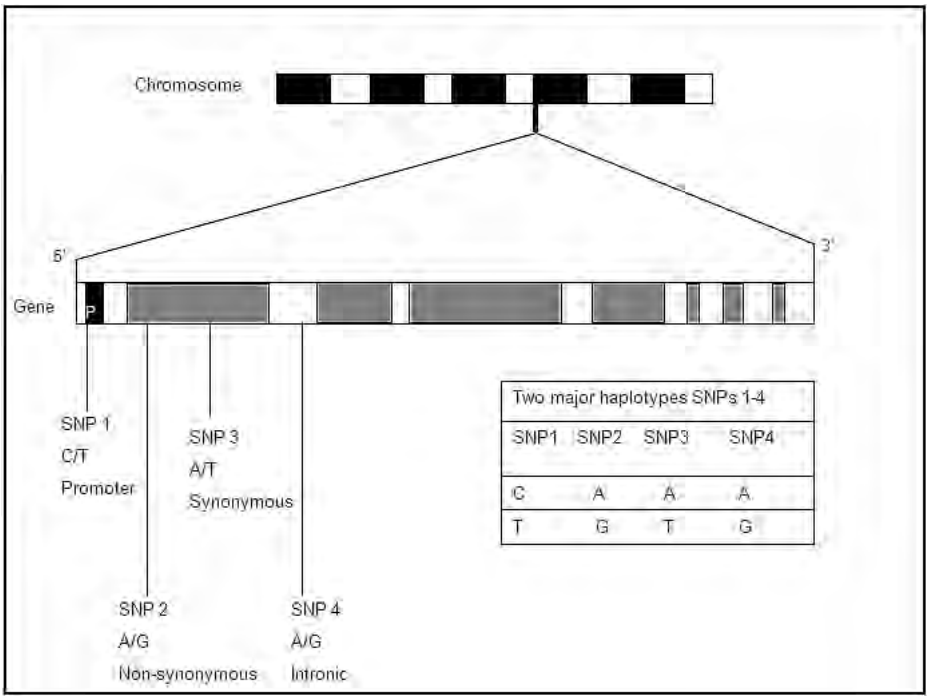


Figure 8. Haplotypes and linkage disequilibrium (LD) in SNP selection [Figure modified from (Tabor *et al.*, 2002)]. The figure represents a set of consecutive SNPs 1-4 cluster together on a chromosome which is inherited as a block so called haplotype. SNPs 1-4 are in LD and form two common haplotypes "CAAA" and "TGTG" which can be identified by the genotype of one of the SNPs and tested for association indirectly as it is in LD with the other three SNPs.

A *genome-wide association study* (GWAS) is based on a large amount of genotyping, including hundred of thousands, or even up to a million SNPs distributed throughout the genome. The GWAS era arrived with the proposed hypothesis of the "common-disease common-variant" (Lander, 1996). This theory suggests that the susceptibility genes in common complex diseases represent common SNPs of small effects but with high frequency in the general population. Information on LD is used to select a non-redundant set of SNPs for genotyping so that approximately 80% SNPs of the genome variation is tagged by the GWAS platforms (Kruglyak, 2008). Thus far, GWA studies conducted on MDD, (Sullivan *et al.*, 2009, Lewis *et al.*, 2010, Muglia *et al.*, 2010, Wray *et al.*, 2010, Shi *et al.*, 2011, Shyn *et al.*, 2011), have failed to achieve genome-wide significance. This is ascribed to inadequate sample sizes, genetic heterogeneity, and inadequate effect size of the risk variants, which together results in insufficient statistical power.

3 AIMS OF THE STUDY

Depression comprises a variety of symptoms, including early morning awakenings and fatigue, which are also signs of disturbed sleep. Several neurobiological mechanisms have been indicated in regulation of sleep and induction of depression. Presence or absence of disturbed sleep symptoms may reflect differences in the neurobiological processes that lead to depression. The main aim of this study was to identify genetic variants predisposing to depression and disturbed sleep in the Finnish population by using a candidate gene strategy.

The specific aims of this thesis were:

I. To examine the association of polymorphisms in genes that are functionally related to serotonergic and glutamatergic neurotransmission systems, to neural plasticity and to the HPA-axis, in depression and disturbed sleep.

II. To examine the association of polymorphisms in genes involved in the circadian system in depression and disturbed sleep, and in seasonal variations in mood and behavior.

III. To elucidate the role of genetic variants associated with depression and disturbed sleep (from studies I and II) in the regulation of sleep in healthy individuals.

4 SUBJECTS AND METHODS

4.1 Study subjects

The subjects of this thesis study were recruited from a cross-sectional, nation-wide population-based health interview and examination survey Health 2000 (studies I-III) and FINRISK study 2007 survey (study III). Detailed information about these two cohorts can be found at (http://www.terveys2000.fi/indexe.html) and (http://www.ktl.fi/portal/4168). The Institutional Review Board of the Helsinki and Uusimaa Hospital District approved the study. All participants provided full informed consent for the collection of blood samples and subsequent analyses.

Subjects from Health 2000 program

The Health 2000 program is a nationwide population-based health survey carried out in Finland between the fall of 2000 and spring of 2001 by the National Institute for Health and Welfare (THL). Nationally representative samples of 10,000 individuals have been drawn from the population aged 18 and over. The aim of the Health 2000 program was to create a solid informational basis on the state of health and disease in Finland (Aromaa and Koskinen, 2004). The health status of all study subjects was evaluated by an interview conducted at home and a health examination at the local healthcare centre. The participants were asked to fill in questionnaires at home and to bring them along to the local healthcare center for a health examination. The health interview included questions on previous illnesses, healthcare use, medications, living environment, and socioeconomic factors. The trained nurses and physicians of five university hospital divisions (1: Helsinki and Uusimaa, 2: Varsinais-Suomi, 3: Pirkanmaa, 4: Pohjois-Savo, 5: Pohjois-Pohjanmaa) monitored the health status of all subjects. The health examination comprised standardized symptom interviews, laboratory sampling, blood pressure measurement, and mental health interviews. The stages of the health examination are summarized in table 9. To maximize the participation rate in the survey, advertisements in the media, telephone calls, interviews at home, and mailed reminders were used. Of the study population aged 30 and over, 89% participated in the interviews at home and 85% in the health examination. Of the study population's young adults aged 18 to 29 years, 80% were interviewed, which included the symptom interview (Aromaa and Koskinen, 2004).

Table 9. Flow chart of the health examination in the Health 2000 survey [Adapted from (Aromaa and Koskinen, 2004)].

Time	*Activities By Field Personnel*
AT HOME	
90 minutes	INTERVIEW (by Statistics Finland's interview organization)
30 minutes	FILLING IN QUESTIONNAIRE 1 [Questionnaire 1 retrieved information on: functional capacity and quality of life, use of time and leisure activities, physical activity, alcohol consumption, mental health as well as perceived strain at work and burnout]
AT HEALTHCARE CENTRE (3hours and 15 minutes)	
15 minutes	1 RECEPTION - information, informed consent, symptom interview [covered respiratory and cardiovascular symptoms, allergies and musculoskeletal symptoms] - handing Questionnaire 2 and the urine sample container
15 minutes	2 MEASUREMENTS: height, body circumference, ECG, blood pressure
15 minutes	3 MEASUREMENTS: spirometry, bioimpedance, heel bone density
15 minutes	4 LABORATORY - drawing blood samples (100 ml), handling of samples
15 minutes	5 ORAL EXAMINATION - clinical oral examination, orthopantomography
15 minutes	SNACK, FILLING IN QUESTIONAIRE 2 [Questionnaire 2 retrieved information on: gastrointestinal diseases, respiratory diseases and vaccinations]
30 minutes	6 FUNCTIONAL CAPACITY TESTS - physical and cognitive capacity, vision and hearing
30 minutes	7 CLINICAL EXAMINATION
30 minutes	8 MENTAL HEALTH INTERVIEW
15 minutes	9 FINAL INTERVIEW - checking that all examinations and questionnaires have been completed - handing Questionnaire 3 and Dietary Questionnaire - information about the previous and possible further examinations

AT HOME (100 minutes)	(HEALTH EXAMINATION FOR THOSE NOT ATTENDING THE HEALTH EXAMINATION PROPER AT THE HEALTHCARE CENTRE ETC.)
40 minutes	FILLING IN QUESTIONNAIRE 3 [Questionnaire 3 retrieved information on: sleep and sleeping, seasonal variation, mental health problems, emotions and feelings, health-related quality of life, driving, alcohol, attitudes regarding health etc.] FILLING IN DIETARY QUESTIONNAIRE [Dietary questionnaire retrieved information on: milk products, cereal products, vegetables, meat and fish dishes fruits and beverages]
AT UNIVERSITY HOSPITALS AND RESEARCH INSTITUTES	FURTHER EXAMINATIONS FOR SUBSAMPLES
FROM REGISTERS	REGISTER DATA

The details of sampling design, target population, and methods of the survey have been reported elsewhere (http://www.ktl.fi/terveys2000/index.uk.html).

***Health 2000*.** In studies I and II, 1654 subjects (967 females and 687 males) with a minimum age of 30 years were included from the Health 2000 survey. Of these subjects, 384 belonged to the depression group (D+) (259 females, mean age 49 years, and 125 males, mean age 48 years). Among these cases, 109/259 females and 61/125 males also reported early morning awakenings (group D+EMA+). The corresponding numbers for fatigue were 194 and 103 (group D+FAT+), whilst 94 females and 58 males belonged to both groups, D+EMA+ and D+FAT+.

In the control group (D–), there were 1270 individuals (708 females, mean age 46 years, and 562 males, mean age 45 years) with no depression or any other psychiatric disorders. The controls were matched by their age and gender to the cases (n=384) and comprised 392 additional individuals from the general population who did not have any sleep related problems or mental disorders. Altogether the control groups included 705 females and 561 males with no signs of early morning awakenings (group D-EMA-), and 580 females and 482 males with no signs of fatigue (group D-FAT-). Moreover in study II, information on the global seasonality scores (GSS) was available for 967 females and 687 males from the total sample of 1654 subjects.

***Genmets (D-) sample*.** In study II, a secondary or independent sample set drawn from the Health 2000 program was included, which was originally selected for a study on metabolic disorders. Of these samples, 141 cases had a CIDI-based diagnosis of depression. They were excluded and referred to as the Genmets (D-) sample. Within the secondary sample, the genetic information was available for 1512 individuals (759 females, mean age 53 years, and 753 males, mean age 47 years). There

were 314 females and 311 males with a metabolic syndrome, which was controlled for as a covariate status in the analyses. Furthermore, 695/759 females and 691/753 males had provided information on the GSS. The corresponding numbers for other studied phenotypes in the Genmets (D-) sample are presented in table 10.

Subjects from FINRISK study 2007 survey

This survey was carried out in 2007 in Finland for the study of chronic diseases, coronary risk factors, and health behaviour (http://www.ktl.fi/portal/4168). The first phase of the study took place from January to March and involved a total of 6258 subjects (a response rate of 63%) (Konttinen *et al.,* 2010). The second phase of this study survey was conducted from April to June 2007, the goal of which was to investigate the Dietary, Lifestyle and Genetic Determinants of Obesity and Metabolic Syndrome (DILGOM sub study). Of the 5024 individuals who took part in this phase of the study, the response was 84% (Konttinen *et al.,* 2010). This study included 655 healthy individuals from the Helsinki-Vantaa region, originally matched as controls for a cardiovascular risk factor study.

Combined sample set **(Health 2000 program and FINRISK study 2007 survey).** Altogether 3147 healthy individuals (1626 females, mean age 51 years, and 1521 males, mean age 49 years) were included in study III, which were pooled from the following three subsamples with information on sleep duration (Table 10):

- 1135 individuals (610 females and 525 males) from the studies I and II with no depression and no complaint of disturbed sleep (Health 2000, Control group).
- 1357 individuals (690 females and 667 males) from the Genmets (D-) sample, from study II.
- 655 healthy individuals (326 females and 329 males) from the national FINRISK study 2007 survey.

Table 10. Characteristics of study subjects.

Study	*Sample set*	*Study phenotypes*	*Study subjects*			
I & II	Health 2000	***Dichotomized variables***				
		Cases	***Females***	***Age (Average ± SD)***	***Males***	***Age (Average ± SD)***
		Depression (D+) (All)	259	49.02±13.65	125	47.94±10.75
		Early morning awakenings (D+EMA+)	109[a]	51.64±13.19	61[b]	48.85±9.85
		Fatigue (D+FAT+)	194[a]	50.10±14.06	103[b]	48.39±11.07
		Controls				
		No depression (D-) (All)	708	46.35±11.80	562	44.80±10.57
		Controls without early morning awakenings (D-EMA-)	705	46.32±11.80	561	44.83±10.57
		Controls without fatigue (D-FAT-)	580	46.10±11.68	482	45.28±10.69
II	Genmets (D-) sample	***Additional Controls***				
		Controls with early morning awakenings (D-EMA+)	274[c]	56.87±10.38	248[d]	54.29±10.30
		Controls with fatigue (D-FAT+)	388[c]	56.24±10.22	342[d]	48.56±11.42
		Controls without early morning awakenings (D-EMA-)	412	69.05±7.77	419	48.82±11.84
		Controls without fatigue (D-FAT-)	349	56.97±10.48	383	53.08±10.64
III	Combined sample set	***Quantitative traits***				
		Sleep duration (All)	1626	51 ± 13	1521	49 ± 13
		Short sleepers	195	54 ± 15	234	49 ± 11
		Midrange sleepers	1220	50 ± 13	1156	48 ± 13
		Long sleepers	211	47 ± 16	131	55 ± 14

Overlap in the Health 2000 samples with D+EMA+ and D+FAT+ was [a]94 for females and [b] 58 for males. Overlap in the Genmets (D-) sample with D-EMA+ and D-FAT+ was [c]165 for females and [d]136 for males.

4.2 Phenotyping methodology

Only individuals that answered the diagnostic questions regarding the MDD, disturbed sleep, and the sleep duration, were included in this study.

The composite international diagnostic interview (I-II)

In studies I and II, a Finnish translation of the German, computerized version of the Composite International Diagnostic Interview (M-CIDI) was used (Pirkola *et al.*, 2005). This program uses operationalized criteria for Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) diagnoses and allows estimation of DSM-IV diagnoses for major mental disorders (Pirkola *et al.*, 2005). In this study, a 12-month prevalence of major depressive episodes was used as a diagnostic, in addition to dysthymic disorder (see in section 2.1.2.1). The interviewers were non-psychiatric health care professionals who had been trained for 3-4 days by psychiatrists and physicians who had been authorized by the World Health Organization (WHO) (Ahola *et al.*, 2005). The individuals with no complaints of disturbed sleep, and no depression or any other psychiatric disorder according to the CIDI using the DSM-IV criteria (American Psychiatric Association, 1994), were referred to as healthy individuals or controls (group D–) in studies I-III.

Assessment of early morning awakenings and fatigue (I-II)

Symptoms of early morning awakenings (EMA) and fatigue, which would point to disturbed sleep in depression, were questioned systematically in the interview (Table 11). Those who agreed to the scored question “Do you wake up in the middle of sleep very early in the morning?” (KYS3_K03), and felt that this happened either “often” or “almost every night” were coded as positive for the EMA feature. For the assessment of fatigue, three different items from the questionnaire were clustered into a single item in order to have a more robust estimation of fatigue. Those who felt either (i) “nearly always” or “often more tired than generally people of the same age” (KYS3_K04) or (ii) “powerless”, “fatigued” (KYS1_K1305) or “overstrained”, “burned-out” (KYS1_K1307) to some extent, quite a lot, or very much during the last 30 days, were coded as positive for the feature of fatigue.

Table 11. Questionnaire for assessment of EMA and fatigue.

Phenotype	*Question*	*Explanation*	*Coding YES*	*Coding NO*
Early morning awakenings (EMA)	KYS3_K03: Do you wake up in the middle of sleep very early in the morning?	1= Not at all 2 = Now and then 3 = Often 4 = Almost every night	3,4	1,2
Fatigue	*KYS3_K04: Do you consider yourself to be fatigued more often than other people of the same age during the daytime?	1 = Yes, nearly always 2 = Yes, very often (at least weekly) 3 = No 4 = I can not say	KYS3_K04 = 1,2 KYS1_K1305 = 3,4,5 KYS1_K1307 = 3,4,5	KYS3_K04 = 3,4 KYS1_K1305 = 1,2 KYS1_K1307 = 1,2
	*KYS1_K1305: Powerless or fatigued *KYS1_K1307: Over-strained or burned-out	1 = Not at all 2 = A little 3 = To some extent 4 = Quite a lot 5 = Very much		

*1 missing value allowed; if ≥2 missing values and the criteria for "YES" are not fulfilled, item encoded as "UNKNOWN".

Assessment of global seasonality score (GSS) (II)

Study participants also completed the Seasonal Pattern Assessment Questionnaire (SPAQ) (Rosenthal NE *et al.*, 1984); a self-report instrument for the assessment of seasonal changes in the length of sleep, social activity, mood, weight, appetite, and energy level whose sum yields the global seasonality score (GSS) (Lahti *et al.*, 2008). These items were scored ranging from 0 to 24; over 10 or more points indicate greater seasonality changes. A seasonal variation in mood and behaviour was measured with a two-factor solution for the global seasonality score (GSS). Factor 1 was considered as a metabolic factor (GSSf1) including items on weight and appe-

tite, and factor 2 as a mental factor (GSSf2) including items concerning sleep duration, social activity, mood, and energy level (Rintamaki *et al.*, 2008).

Sleep duration (III)

Sleep duration in healthy subjects was assessed using the self-reported question (KYS3_K01) "How many hours do you sleep on average during 24 hours?" The participants were classified according to their duration of sleep as 'short sleepers (6 hours or less)', 'midrange sleepers (7-8 hours)', and 'long sleepers (9 hours or more)'.

4.3 Candidate genes and marker selection

Study I focused on the candidate genes from the serotonergic (*TPH2*, *SLC6A4*, *COMT* and *MAOA*), glutamatergic (GAD1, *P2RX7*, *DAOA* and *GRIA3*), neural plasticity (*DISC1*, *CREB1*, *NTRK2 and BENF*), and HPA-axis systems (*NR3C1* and *CRHR1*). The polymorphisms of the candidate genes investigated are listed in table 12.

Table 12. List of the studied candidate genes and the SNPs in study I.

Gene Symbol	*Chr*	*SNP*	*Position*	*Role*	*A.A*	*Alleles*
Serotonergic system						
TPH2	12q21.1	rs11178997	70618420	Promoter	-	T/A
		rs10748185	70622122	Intron	-	A/G
		rs1386492	70648532	Intron	-	T/C
		rs7305115	70659129	Exon	PRO/ PRO	G/A
		rs12229394	70679181	Intron	-	G/A
		rs4760820	70683263	Intron	-	C/G
		rs10879354	70696049	Intron	-	T/C
		rs1487275	70696559	Intron	-	A/C
		rs4290270	70702502	Exon	ALA/ ALA	T/A
SLC6A4	17q11.2	rs3794808	25555919	Intron	-	C/T
		rs140700	25567515	Intron	-	C/T
		Intron 2 VNTR (STin2VNTR)	25572535	Intron	-	9R/10R/ 12R
		rs6354	25574024	Exon	-	T/G
		rs4251417	25575984	Intron	-	C/T
		5-HTTLPR	25588443	Promoter	-	L/S
		rs25531	28564350	Promoter	-	A/G

COMT	22q11.21	rs4680	18325825	Exon	VAL/ MET	A/G
		rs165599	18331335	3' UTR	-	A/G
MAOA	Xp11.3	*MAO-A-30 bp VNTR*	43270603	Promoter	-	3R/3.5R /4R /5R
		rs1465108	43294463	Intron	-	G/A
		rs5906957	43303564	Intron	-	G/A
		rs6323	43347290	Exon	ARG/ ARG	T/G
Glutamatergic system						
GAD1	2q31	rs12185692	171496333	Promoter	-	C/A
		rs2241165	171503886	Intron	-	T/C
		rs2058725	171515628	Intron	-	T/C
		rs769407	171519215	Intron	-	G/C
		rs3791850	171533607	Intron	-	G/A
P2RX7	12q24	rs591874	120034185	Intron	-	A/C
		rs1718125	120055739	Intron	-	C/T
		rs208290	120056776	Intron	-	G/A
		rs208294	120062973	Intron	-	C/T
		rs504677	120067909	Intron	-	C/T
		rs1718119	120077823	Exon	-	G/A
		rs2230912	120084916	Exon	-	A/G
DAOA	13q34	rs2391191	104917447	Exon	ARG/ LYS	G/A
		rs12874006	104918986	Intron	-	G/T
		rs1539070	104922458	Intron	-	C/G
		rs2153674	104929139	Intron	-	C/T
		rs701567	104939996	Intron	-	T/C
		rs12864685	104941856	3' UTR	-	C/T
		rs778326	104948042	3' UTR	-	A/T
		rs954580	104949855	3' UTR	-	T/C
		rs778330	104952561	Intron	-	A/G
		rs778336	104954515	Intron	-	C/T
		rs778293	104967200	Intron	-	T/C
GRIA3	Xq25	rs6649004	122204322	Intron	-	T/C
		rs5911611	122237152	Intron	-	G/A
		rs526716	122272504	Intron	-	C/T
		rs3848874	122298920	Intron	-	G/A
		rs687577	122304639	Intron	-	C/A
Neural Plasticity system						

DISC1	1q42.1	rs751229	228075274	Intron	-	T/C
		rs3738401	228137030	Exon	ARG/ GLN	G/A
		rs821616	228451333	Exon	SER/ CYS	A/T
		rs1411771	228481510	Exon	-	T/C
CREB1	2q34	rs2709356	208237598	Intron	-	C/T
		rs10932201	208251763	Intron	-	G/A
		rs11904814	208252304	Intron	-	T/G
NTRK2	9q22.1	rs1025743	84544671	Intron	-	C/T
		rs1187362	84567821	Intron	-	A/T
		rs4144551	84599165	Intron	-	A/G
		rs3654	84660045	Intron	-	T/C
		rs7038236	84717956	Intron	-	C/A
		rs11140793	84721034	Intron	-	A/C
		rs1221	84722164	Intron	-	C/T
		rs3739570	84867132	3' UTR	-	G/A
BDNF	11p13	rs2203877	27627486	3' UTR	-	C/T
		rs6265	27636492	Exon	VAL/ MET	C/T
		rs11030102	27638172	Intron	-	C/G
		rs11030108	27652040	Intron	-	G/A
		rs6484320	27659764	Intron	-	A/T
		rs1491850	27706301	Promoter	-	T/C
		rs1491851	27709339	Promoter	-	C/T
HPA-axis system						
NR3C1	5q31.3	rs9324916	142702853	Intron	-	C/G
		bclI	142758768	Intron	-	G/C
		rs10052957	142766894	Promoter	-	C/T
CRHR1	17q21.31	rs173365	41256855	Intron	-	G/A
		rs16940665	41263677	Exon	THR/ THR	T/C
		rs1876828	41267306	Intron	-	C/T

Chr-Chromosome, SNP-Single nucleotide polymorphism, A.A-Amino acids. SNPs information from the NCBI dbSNP BUILD 125.

Study II focused on candidate genes from the circadian system. Altogether 18 candidate genes were included. The polymorphisms of the candidate genes investigated are listed in table 13.

Table 13. List of the studied candidate genes and the SNPs in study II.

Gene Symbol	*Chr*	*SNP*	*Position*	*Role*	*A.A*	*Alleles*
PER3	1p36.23	rs3753503	7764558	Promoter	-	G/A
		rs228729	7768282	Intron	-	C/T
		rs228682	7778933	Intron	-	T/C
		rs228642	7785880	Intron	-	T/C
		rs1891217	7794436	Intron	-	C/T
		rs17374292	7803821	Intron	-	C/T
		rs12035969	7815433	Intron	-	C/T
		rs10462021	7819720	Exon	HIS/ ARG	A/G
PER2	2q37.3	rs881933	238815780	Downstream	-	C/G
		rs934945	238819792	Exon	GLY/ GLU	C/T
		rs6431590	238829867	Intron	-	T/C
		rs3739064	238841125	Intron	-	A/G
		rs11894535	238841812	Intron	-	G/A
		rs10462023	238849320	Intron	-	C/T
		rs2304672	238851328	5’ UTR	-	C/G
		rs4663302	238868500	Promoter	-	C/T
NPAS2	2q11.2	rs6722909	100827527	Intron	-	A/G
		rs1811399	100845446	Intron	-	A/C
		rs12712083	100875331	Intron	-	A/G
		rs2117714	100888209	Intron	-	A/G
		rs6725296	100904136	Intron	-	G/A
		rs3820785	100914246	Intron	-	A/G
		rs12712085	100931583	Intron	-	A/G
		rs17025078	100946008	Intron	-	G/A
		rs2305160	100957736	Exon	THR/ ALA	G/A
		S471L	100960623	Exon	SER/ LEU	C/T
		rs1374324	100980754	3' end	-	G/A
CLOCK	4q12	rs10462028	55993057	Downstream	-	G/A
		rs1801260	55996126	3' UTR	-	A/G
		rs11932595	56018354	Intron	-	T/C
		rs6850524	56076754	Intron	-	C/G
		rs4864548	56108560	Promoter	-	G/A
NFIL3	9q22.31	rs1619450	93208976	3' UTR	-	T/C
		rs10991925	93217461	Intron	-	A/G

		rs2440589	93218808	Intron	-	G/A
		rs968821	93227455	Promoter	-	C/G
		rs813498	93228207	Promoter	-	T/C
BHLHE40	3p26	rs908078	4999771	Exon	GLY/ GLY	T/C
		rs11130215	5001008	Exon	-	A/G
		rs2137947	5005961	3' UTR	-	T/C
CRY2	11p11.2	rs10838524	45826753	Intron	-	A/G
		rs7123390	45847994	Intron	-	G/A
		rs10838527	45859770	3' UTR	-	A/G
		rs3824872	45862181	Downstream	-	C/A
ARNTL	11p15.2	rs2279287	13255061	Promoter	-	C/T
		rs7950226	13274715	Intron	-	A/G
		rs10766074	13275142	Intron	-	T/C
		rs1982350	13306707	Intron	-	G/A
		rs6486121	13312346	Intron	-	C/T
		rs1562438	13320776	Intron	-	C/T
		rs2290036	13336364	Intron	-	T/C
		rs1868049	13340258	Intron	-	C/T
		rs11022778	13347436	Intron	-	T/G
		rs3816358	13348048	Intron	-	C/A
		rs4757151	13348789	Intron	-	G/A
		rs2278749	13354454	Intron	-	T/C
		rs3897902	13358379	Intron	-	A/C
		rs969485	13359619	Intron	-	A/G
ARNTL2	12p11.23	rs10842905	27374170	Promoter	-	T/C
		rs7137588	27375106	Promoter	-	C/G
		rs4964052	27380597	Intron	-	G/T
		rs922270	27396218	Intron	-	T/C
		rs17413842	27414190	Intron	-	A/G
		rs4964060	27424634	Intron	-	G/A
		rs7304939	27435612	Intron	-	C/T
		rs12299407	27440450	Intron	-	T/C
		rs1037921	27444833	Exon	ASN/ SER	A/G
		rs4931075	27456413	Intron	-	G/A
		rs2289709	27464900	Downstream	-	C/T
BHLHE41	12p12.1	rs1048155	26164355	3' UTR	-	C/G
TIMELESS	12q13.2	rs2291739	55100920	Exon	PRO/ LEU	T/C

		rs2291738	55101548	Intron	-	G/A
		rs7486220	55123683	Intron	-	G/A
		rs1082214	55132757	Promoter	-	C/T
CRY1	12q23.3	rs2287162	105905035	Downstream	-	C/G
		rs2287161	105905270	Downstream	-	G/C
		rs10861683	105905690	Downstream	-	T/A
		rs11113179	105976915	Intron	-	G/A
		rs3809237	106011042	5' UTR	-	A/G
RORA	15q22.2	rs2290430	58579246	Intron	-	A/C
		rs12914584	58605378	Intron	-	A/C
		rs2028122	58628567	Intron	-	G/A
		rs4775281	58631063	Intron	-	T/G
		rs4774370	58680723	Promoter	-	T/C
		rs1863270	58799551	Intron	-	T/G
		rs11637301	58835190	Intron	-	T/A
		rs341373	58900506	Intron	-	G/A
		rs8027829	58961163	Intron	-	C/T
		rs16943429	59066545	Intron	-	A/G
		rs1568717	59149739	Intron	-	G/T
		rs893287	59181524	Intron	-	C/T
		rs4774388	59254290	Intron	-	A/G
		rs1816624	59256614	Intron	-	A/G
		rs6494251	59301859	Intron	-	C/T
		rs10438343	59310872	Promoter	-	G/A
TIPIN	15q22.31	rs3759785	64412524	3' UTR	-	G/A
		rs3759786	64416457	Exon	ALA/ SER	G/T
		rs2063690	64428786	Exon	ALA/ GLY	C/G
		rs8031897	64433145	Intron	-	T/C
NR1D1	17q11.2	rs2314339	35506738	Intron	-	G/A
		rs2071427	35508018	Intron	-	G/A
		rs2269457	35508215	Intron	-	T/C
		rs2071570	35510616	Promoter	-	C/A
PER1	17p13.1	rs2289591	7988735	Intron	-	G/T
		rs2253820	7988894	Exon	THR/ THR	G/A
		rs3027188	7989710	Intron	-	G/C
		rs885747	7991462	Intron	-	G/C
		rs2518023	7997331	Promoter	-	G/T

DBP	19q13.33	rs3745733	53824446	Exon	-	G/A
CSNK1E	22q13.1	rs135745	37013583	3' UTR	-	G/C
		rs2075984	37020835	Intron	-	A/C
		rs5750581	37025352	Intron	-	T/C
		rs7289981	37049908	Promoter	-	C/T

Chr-Chromosome, SNP-Single nucleotide polymorphism, A.A-Amino acids. SNPs information from the NCBI dbSNP BUILD 125 and 129.

In study III, we examined 23 SNPs from 12 candidate genes; *TPH2* (rs12229394), *SLC6A4* (rs4251417), *GRIA3* (rs687577, rs526716), *DISC1* (rs3738401), BDNF (rs6265, rs1491850), *CRHR1* (rs173365), *NPAS2* (rs12712083), *NFIL3* (rs1619450), *ARNTL* (rs6486121, rs3816358, rs969485), *ARNTL2* (rs4964060, rs7304939, rs1037921, rs2289709), *TIMELESS* (rs2291738, rs1082214), and *RORA* (rs4774370, rs8027829, rs1568717, rs4774388) which associated with depression and disturbed sleep (Pointwise $P<0.05$) in the two previous studies (I and II).

Marker selection (I-III)

The selection of haplotype tagging SNPs was based on the Centre d'Etude du Polymorphisme Humain (CEPH) genotype data of the International HapMap Project (The International HapMap Consortium, 2005), using a tool Tagger implemented in Haploview program (V.4.0) (Barrett *et al.*, 2005). The tagging SNPs were selected with a cut-off value for r^2 of 0.8 and minor allele frequency of 0.2 (study I), or 0.1 (study II). For the largest genes (*NTRK2*, *NPAS2*, and *RORA*), SNPs were selected evenly spaced throughout the gene with the same threshold values.

4.4 Genotyping

DNA extraction (I-III)

Genomic DNA was extracted from ethylenediaminetetraacetic acid (EDTA)-treated peripheral blood leukocytes following the standard manufacturer's protocol for the 'PureGene DNA isolation kit' (Gentra Systems, Minneapolis, Minnesota, USA). DNA samples were stored at -70^{o}C before they were used.

Sequenom's MassARRAY platform

Genotyping of SNPs in the Health 2000 sample (studies I-III) was performed at the Finnish Genome Center in Helsinki using Sequenom's MassARRAY with iPLEX

Gold and MALDI-TOF (matrix-assisted laser desorption-ionization-time of flight) mass spectrometry, following the manufacturer's guidelines (Sequenom Inc., San Diego, CA, USA). Allele-specific primers for the SNP assays were designed using the Sequenom's MassARRAY Assay Designer software (V.3.1) (Sequenom Inc., San Diego, CA, USA). As quality controls, eight duplicated DNA samples and eight water controls were included in each plate. The qualities of genotype spectra were analyzed using MassARRAY Spectro TYPER software (V.2.0 and 4.0), and verified manually.

Repeat length polymorphisms and RFLP genotyping

Repeat length polymorphisms from the *SLC6A4* promoter region (*5-HTTLPR*), from intron 2 (*STin2 VNTR*), and from the promoter region of *MAOA* (*MAO-A-30bp VNTR*) were genotyped in study I. The PCR primers were designed with the Primer3 program (Rozen and Skaletsky, 2000). SNP rs25531, located next to the *5-HTTLPR*, was also genotyped using the *Msp*I restriction enzyme on the *5-HTTLPR* PCR product, which resulted in the cutting of the long allele (L) into fragments of 150 and 99 bp in case that the minor allele (G) of rs25531 was present in the sequence (Chorbov *et al.*, 2007). The PCR products were electrophoresed on an ABI3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and the alleles automatically identified using an ABI GENEMAPPER (V.4.0) and then verified manually.

Illumina 610 K platform

Genotypes from the Genmets (D-) and FINRISK 2007 samples (studies II and III) were obtained from a GWAS dataset, which was originally genotyped by means of the Illumina 610K chip (Illumina HumanHap 610-Quad SNP array, San Diego, CA, USA) at the Wellcome Trust Sanger Institute, UK.

Quality control

For studies I and II the overall average genotyping success rate for the SNP data was $\geq$ 95%. The Hardy-Weinberg equilibrium with a cut-off of $P<0.05$ was applied by using the Haploview program (V4.1) (Barrett *et al.*, 2005). In studies I and II, ten SNPs failed the HWE test and were excluded from further analyses. When genotyping with the Illumina 610K platform (studies II and III), the call rate for both individuals and markers was >95%, while the markers with MAF <1% or HWE $p<1x10^{-6}$ were excluded.

4.5 Statistical analyses

Association analyses

In studies I and II, a single SNP association test was performed to compare the allele frequencies between cases and controls (dichotomous outcome variables) by using the chi-square test. In study II, a linear regression model was also used to analyze GSS (a quantitative variable). Age and status for metabolic disorder were used as covariates. To investigate the statistical power available for significantly associated variants in studies I and II, the power at $\alpha=0.05$ was calculated using a web-based genetic power calculator (Purcell *et al.*, 2003). In study III, association analysis to self-reported habitual sleep duration (a quantitative variable) was performed separately in females and males using a linear regression model, applying age and metabolic disorder status as covariates. A logistic regression model was then used to test the following groups: short sleep duration ($\leq$ 6 hours) vs. midrange sleep duration (7-8 hours), and long sleep duration ($\geq$ 9 hours) vs. midrange sleep duration (7-8 hours). All analyses were conducted separately for females and males using the PLINK software package, web-based version 1.00 and 1.06 (Purcell *et al.*, 2007).

In case-control comparisons (studies I and II), the following groups were tested: (1) all depressed patients against all controls (D+ vs. D-), (2) depressed patients with early morning awakenings against controls without early morning awakenings (D+EMA+ vs. D-EMA-), and (3) depressed patients with fatigue against controls without fatigue (D+FAT+ vs. D-FAT-). In addition, three multiallelic markers were analyzed in study I. Two different approaches were used to analyze *5-HTTLPR*: (1) the S allele and L allele analyzed separately *(5-HTTLPR*^{1}), (2) grouping the S allele and L allele together (*5-HTTLPR*^{2}), taking into account that the expression of the L allele is equivalent to that of the S allele (Hu *et al.*, 2006). The intron 2 VNTR (*STin2 VNTR*) was analyzed by grouping the 9R and 10R repeat alleles together and comparing them to 12R (Fan and Sklar, 2005). For *MAO-A-30 bp VNTR* analysis, alleles were divided in two groups with high transcription activity allele (3.5 R and 4R), and low transcriptional activity allele (3R and 5R) (Sabol *et al.*, 1998).

Descriptive analyses in studies I and II. After the initial analyses, variants that had given associations with pointwise $P<0.05$ were selected for more detailed analyses in order to test for differences in allele frequencies between the different groups of cases with depression, and with disturbed sleep using PLINK (Purcell *et al.*, 2007). The following non-overlapping groups were examined: (1) D+EMA-FAT- (n=41 females and 16 males), (2) D+EMA+FAT+ (n=94 females and 58 males), (3) D+EMA+FAT- (n=15 females and 3 males; owing to the small number of males we

did not examine their allelic frequencies), (4) D+EMA-FAT+ (n=91 females and 33 males), and controls, D-EMA-FAT- (n=578 females and 481 males).

Analysis of interaction. In study II, SNP-SNP interactions were examined for variants that gave the strongest evidence of association to depression with disturbed sleep. The interaction of gender with the best-associated SNPs was also explored using the logistic regression model with age as covariate.

Corrections for multiple testing. Associations in studies I, II, and III were adjusted for controlling false-positive results by using the permutation and Bonferroni tests. Bonferroni correction is considered a strict method where a significance threshold (alpha=0.05) is divided by the number of all examined models. Therefore, in the primary analyses of studies I-III we used *max (T)* permutation test with 10,000 permutations to obtain empirical corrected P-values. These analyses were conducted using the PLINK software package, web-based version 1.00 and 1.06 (Purcell *et al.*, 2007). The rationale behind the permutation test is that it preserves the LD structure between SNPs which provides a less stringent correction for multiple testing in comparison to the Bonferroni tests that assumes all tests are independent (Purcell *et al.*, 2007).

Linkage disequilibrium (LD) and haplotype-based association analyses

LD between genotyped variants in the candidate genes was estimated using the Haploview program (V.4.0) (Barrett *et al.*, 2005). The PLINK version 1.00 and 1.06 was used for the association analyses of two-SNP (studies I-III), three-SNP (studies II and III), and four-SNP haplotypes (study II) with the sliding window approach (Purcell *et al.*, 2007). SNPs giving an association of $P<0.05$ in the single SNP analysis (studies I and II), and those which survived the multiple correction tests in study III, were included in the haplotype-based association test.

Analysis of transcription factor binding sites

Gender differences can be explained by several molecular and neurobiological mechanisms (see in section 2.1.4). The hormonal regulation of gene expression may play an important role in gender differences in depression and sleep (Dzaja *et al.*, 2005, Bangasser *et al.*, 2010). Therefore, in studies II and III, the transcription factor binding sites of the selected genes were analyzed using the tool ConSite, a platform-independent web resource (Sandelin *et al.*, 2004). The orthologous pairs of genomic sequences were retrieved using a genome browser, EnsEMBL (www.ensembl.org), and the retrieved pairs of genomic sequences were re-aligned using the ORCA aligner (Sandelin *et al.*, 2004).

5 RESULTS AND DISCUSSION

5.1 Contribution of variation in candidate genes from serotonergic and glutamatergic neurotransmission, neural plasticity and the HPA-axis to depression, and disturbed sleep

A large majority of individuals who suffer from depression also have sleep difficulties (Benca *et al.*, 1997), and disturbed sleep has been characterized as one of the diagnostic criteria for depression (American Psychiatric Association, 2000). In fact, several symptoms related to sleep disturbances, including early morning awakenings and fatigue, are frequently found in patients with depression. Accordingly, we hypothesized that the presence or absence of the symptoms and signs of sleep disturbance may also reflect differences in the neurobiological processes underlying depressive disorders. Pathways of serotonergic and glutamatergic neurotransmission are primarily involved in the regulation of wakefulness and REM sleep (Andretic *et al.*, 2008). Animal studies have shown that in many brain regions serotonin and glutamate are present at the highest levels during wakefulness and at the lowest during REM (Portas *et al.*, 2000, Datta *et al.*, 2001). Remarkably, REM sleep alterations are typically observed in depression (Srinivasan *et al.*, 2009). Furthermore, stressful life events can provoke HPA-axis disturbances that play a role in the etiology of depression and induce sleep changes (Wang *et al.*, 2008). Dysfunctioning of neural plasticity is linked to the pathophysiology of MDD and sleep problems (Pittenger and Duman, 2008). Therefore, in the aim of the present study was to examine the role of polymorphisms in such candidate genes, which are functionally related to the serotonergic and glutamatergic neurotransmission systems, to neural plasticity, and the HPA-axis, in the etiology of depression and disturbed sleep. Associations for the allelic variants of 14 candidate genes was assessed using 78 markers in 384 depressed individuals and 1270 controls from the population-based Health 2000 cohort (http://www.terveys2000.fi/indexe.html). The analyses were focused on patients with depression (D+) (n=259 females and 125 males), depressed patients with early morning awakenings (D+EMA+) (n=109 females and 61 males), and depressed patients with fatigue (D+FAT+) (n=194 females and 103 males). The association results of single SNP and haplotype-based analyses are presented in tables 14 and 15.

Several variants of selected functional candidate genes were identified in this work, which could be among the genetic risk factors for depression. In addition, the genetic background for depression without symptoms of disturbed sleep and depression with such symptoms was assessed. These findings show, for the first time, that

some of the genetic risk for depression may actually relate primarily to genetic risk of disturbed sleep, as shown for allelic variations of *TPH2* in females. Others, such as *CREB1* in males, do not show any specificity for sleep related symptoms in their genetic risk for depression.

Amongst the genes that are involved in serotonergic neurotransmission, variants from *TPH2*, *SLC6A*4, *COMT*, and *MAOA* were studied. Rs12229394 from intron 8 of *TPH2* was associated with depression accompanied by fatigue in females (P=0.005, OR=1.40, associating allele 'A'), as did the A-C haplotype of rs12229394 and rs4760820 (P=0.003, OR=1.45). These two SNPs of *TPH2* are tightly linked with each other (LD rs12229394/ rs4760820 D'=0.92, r^2=0.228), as shown in figure 9. The allele 'A' of rs12229394 was also found to associate with fatigue in the control group (D–) (P=0.049, OR=1.34). As signs of poor sleep are well-known risk factors for depression, this finding may reflect that genetic predisposition to fatigue increases the risk of depressive disorders later in life. Previous studies have found evidence for an association of variants from the exon 7 and intron 8 region of the *TPH2* gene with suicide in MDD (Ke *et al.*, 2006, Lopez de Lara *et al.*, 2007), schizoaffective disorder, suicide attempt (Zhou *et al.*, 2005), and with bipolar disorder (Lopez *et al.*, 2007) (Figure 10). Thus, results by others and us support the probability of an involvement of 5' regions of *TPH2* in the regulation of mood (Figure 10).

Table 14. Association results for genes from serotonergic and glutamatergic neurotransmission, neural plasticity, and the HPA-axis with depression and disturbed sleep in females and males.

Chr	*Gene*	*SNP*	*Alleles*	*D+*		*D+EMA+*		*D+FAT+*		*┼MAF (D+)*				*┼MAF (D-)*	*Gender*
				OR	*P-value*	*OR*	*P-value*	*OR*	*P-value*	*EMA-FAT-*	*EMA+FAT+*	*EMA+FAT-*	*EMA-FAT+*	*EMA-FAT-*	
Serotonergic genes															*Females*
17	*SLC6A4*	rs4251417	C/T	1.49	**0.025**	1.31	0.323	1.46	**0.050**	0.10	0.10	0.03	0.12	0.07	
12	*TPH2*	rs12229394	G/A	1.28	**0.023**	1.34	0.059	1.40	**0.005**	0.32	0.36	0.43	0.38	0.30	
Glutamatergic genes															*Females*
2	*GAD1*	rs12185692	C/A	0.75	**0.009**	0.77	0.111	0.74	**0.014**	0.35	0.34	0.40	0.34	0.40	
		rs769407	G/C	1.22	0.071	1.41	**0.023**	1.33	**0.025**	0.26	0.39	0.20	0.31	0.28	
X	*GRIA3*	rs687577	C/A	0.70	**0.046**	0.80	0.424	0.76	0.213	0.03	0.09	0.10	0.08	0.11	
		rs3848874	G/A	1.57	**0.002**	1.70	**0.008**	1.62	**0.002**	0.13	0.17	0.13	0.14	0.10	
Neural plasticity genes															*Females*
1	*DISC1*	rs3738401	G/A	1.30	**0.023**	1.00	0.994	1.28	0.064	0.36	0.26	0.23	0.36	0.25	
2	*CREB1*	rs11904814	T/G	0.85	0.136	0.84	0.279	0.74	**0.021**	0.39	0.31	0.46	0.31	0.38	
11	*BDNF*	rs6265	C/T	0.79	0.123	0.70	0.136	0.66	**0.027**	0.15	0.08	0.30	0.11	0.15	
		rs1491850	T/C	0.76	**0.015**	0.84	0.306	0.79	0.066	0.37	0.40	0.36	0.33	0.43	
HPA- axis genes															*Females*
17	*CRHR1*	rs173365	G/A	1.04	0.728	1.36	**0.041**	1.02	0.841	0.32	0.37	0.36	0.25	0.29	
Glutamatergic genes															*Males*
12	*P2RX7*	rs504677	C/T	0.84	0.223	0.59	**0.010**	0.76	0.088	0.50	0.34	-	0.46	0.46	
13	*DAOA*	rs778330	A/G	1.38	**0.045**	1.37	0.144	1.43	**0.040**	0.30	0.26	-	0.20	0.19	
Neural plasticity genes															*Males*
2	*CREB1*	rs11904814	T/G	0.58	**0.0008**	0.56	**0.008**	0.57	**0.002**	0.20	0.25	-	0.25	0.38	
		rs2709356	C/T	1.46	**0.044**	1.4	0.214	1.42	0.087	0.13	0.18	-	0.17	0.14	

Chr: Chromosome. Alleles: Major/Minor. Bold face P-values show permutation-based P-values< 0.05 [Not Bonferoni-corrected P-values].
┼MAF in non-overlapping group of cases (D+) and controls (D-) based on the presence or absence of EMA or fatigue.
D+EMA+FAT- allele frequencies were not examined in this group due to the small number of males (n=3).

Table 15. Haplotype-based association results given for the haplotype that gave the most significant P-values.

Gene	*SNPs*	*Haplotype*	*Frequency in cases*	*Frequency in controls*	*OR*	*P-values*	*Phenotype*	*Gender*
TPH2	rs12229394-rs4760820	A-C	0.38	0.30	1.45	0.003	D+FAT+	*Females*
GRIA3	rs3848874-rs687577	A-C	0.15	0.10	1.54	0.001	D+FAT+	*Females*
CREB1	rs11904814-rs10932201	G-T	0.25	0.15	1.94	0.003	D+EMA+	*Males*
	rs11904814-rs10932201	G-T	0.25	0.16	1.73	0.001	D+FAT+	*Males*

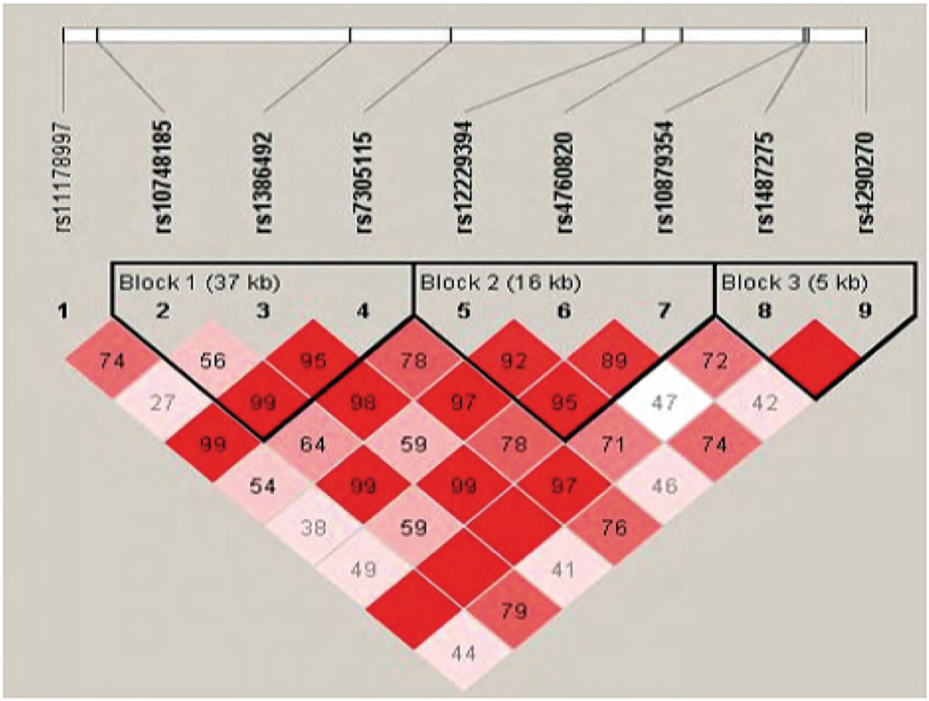


Figure 9. Linkage disequilibrium structure of the *TPH2* SNPs studied in the Health 2000 subjects. The LD and haploblock structure of 9 SNPs of *TPH2* in the study subjects created with Haploview program (Barrett et al., 2005). SNPs are numbered 5' to 3' direction accordingly their relative location. Each square representing a pairwise comparison between two SNPs, LD (D') is shown as different shadings of red darker color indicating higher values of D', up to a maximum of 1. Thick black triangles represent haploblocks, i.e. regions of high LD, between the SNPs. Here blocks are defined using the algorithm solid spine, which identifies the blocks that the first and last SNPs in a block are in strong LD with all intermediate SNPs, but these in-between markers are not necessarily in LD with each other (Barrett et al., 2005). Block 1 includes markers from intron 2, intron 5, and exon 7. Block 2 is located within intron 8, the strongly associated marker, rs12229394, is located within this block. Block 3 includes markers from intron 8 and exon 9.

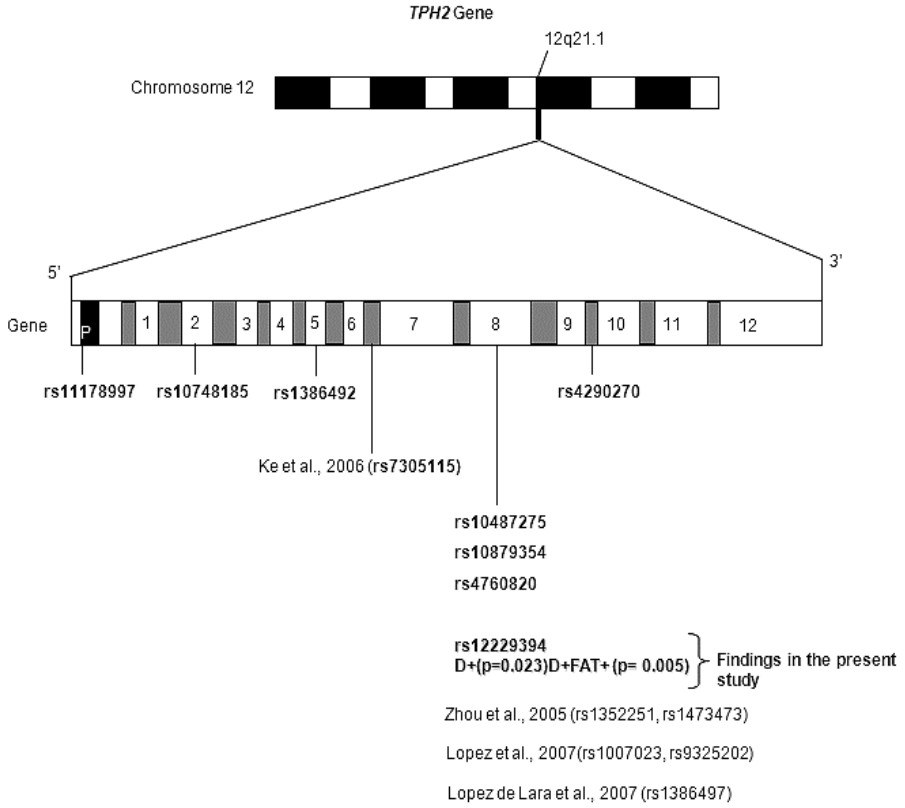


Figure 10. Schematic presentation of the *TPH2* gene. The 5' promoter region (P), 12 introns, and exons (grey blocks) are presented in their approximate locations. SNPs of the *TPH2* gene, which were investigated in the present study, are highlighted with bold face. The figure also summarizes previous evidence for association in the *TPH2* gene, the exon 7 and intron 8 part of the *TPH2* gene is involved in the regulation of mood.

No significant association with a *5-HTTLPR* functional polymorphism in the promoter region of the serotonin transporter gene (*SLC6A4*) was detected, which is consistent with other studies (Ohara *et al.*, 1998, Serretti *et al.*, 2002, Mendlewicz *et al.*, 2004). An allelic variant of *5-HTTLPR* may predispose to depression in individuals with stressful life events (McCaffery *et al.*, 2006, Uher and McGuffin, 2008), but the present study lacked the examination of stressful life events. A trend for association of *SLC6A4* with depression and depression accompanied by fatigue (P=0.025, 0.050, respectively) in females was seen for the SNP rs4251417 in intron 1. A tendency of association was also observed with depression in females for the high-activity allele of *MAOA-uVNTR* (P=0.055, OR=0.80) and allele Met $^{108/158}$ from the functional SNP of *COMT* (P=0.068; OR=0.80). For all of the genes studied from the serotonergic system, associations were observed only with females, which may

relate to a variable regulation of gene expression in the two genders, as expression of serotonin transporter genes is modified by sex hormones (Gubbels Bupp *et al.*, 2008).

Amongst the genes involved in glutamatergic neurotransmission, variants of *GAD1*, *P2RX7*, *DAOA* and *GRIA3* were studied here. The intronic SNP rs3848874 of *GRIA3* associated with depression, depression accompanied by early morning awakenings, and fatigue in females (P=0.002, 0.008 and 0.002, respectively). A major haplotype A-C of rs3848874 and rs687577 of *GRIA3* was associated with depression in females (P=0.001, OR=1.54). *GRIA3* has previously been identified in Italian populations among female patients with schizophrenia (Magri *et al.*, 2008). For *GAD1*, rs12185692 in the promoter region showed evidence of association with depression and depression accompanied by fatigue (P=0.009, 0.014, respectively) in females. Previous studies suggested a role for the promoter region of *GAD1* in bipolar affective disorder (Lundorf *et al.*, 2005), and in schizophrenia (Addington *et al.*, 2005). In addition, variants from other parts of *GAD1* were associated with anxiety and depressive disorders (Hettema *et al.*, 2006). In males, rs778330 from *DAOA* associated with depression and depression accompanied by fatigue (P=0.045, OR=1.38; P=0.040, OR=1.43, respectively), while rs504677 from the *P2RX7* gene showed association with depression accompanied by early morning awakenings (P=0.010, OR=0.59).

Amongst the genes with a function in neural plasticity, *DISC1*, *CREB1*, *NTRK2*, and *BDNF* were studied. Rs2709356 located in intron 1 of *CREB1* showed association with depression in males (P=0.044, OR=1.46), as did allele 'T' of rs11904814 in intron 3 of *CREB1* (P=0.0008, OR=0.58). The same allele associated significantly also with depression accompanied by fatigue in females (P=0.021, OR=0.74). Allelic haplotype G-T of rs11904814 and rs10932201 associated with depression accompanied by early morning awakenings (P=0.003, OR=1.94) and with depression accompanied by fatigue (P=0.001, OR=1.73) in males. These three SNPs of *CREB1* were in the same LD block (LD rs2709356/rs10932201 D'=1.0, r^2=0.144; rs2709356/rs11904814 D'=0.988, r^2=0.091; rs10932201/rs11904814 D'=1.0, r^2=0.518), as shown in figure 11.

CREB1 is a member of the basic leucine zipper family of transcription factors consisting of 341 amino acids (Mayr and Montminy, 2001). The promoter region of this gene has earlier been observed to be associated with depressive disorders in females (Zubenko *et al.*, 2003), and in suicide (Perlis *et al.*, 2007) (Figure 12). Furthermore, the *CREB1* variant from intron 5 also associated with MDD (Dong *et al.*, 2009) (Figure 12). The variant associated with suicide, rs7569963 (Perlis *et al.*, 2007), is in LD with intron 3 SNP rs11904814 (D'=1, r^2=0.892). Zubenko and Huges 3rd reported that G to A transition at position -656 of the promoter region of *CREB1* changed the activity of the promoter in the presence of female gonadal steroids

(Zubenko and Hughes, 2009). Therefore, it is possible that this candidate gene of mood disorders can exert its effect differentially in males and females.

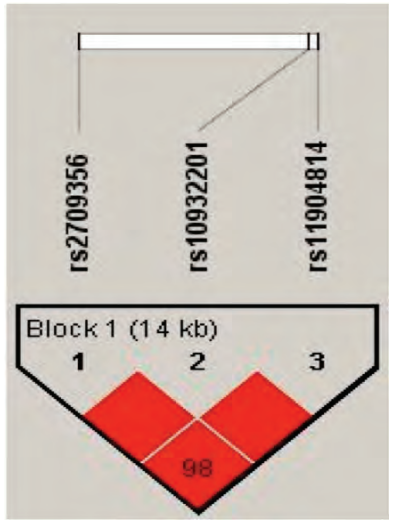


Figure 11. Linkage disequilibrium structure of the *CREB1* SNPs studied in the Health 2000 subjects. The LD and haploblock structure of 3 SNPs of *CREB1* in the study subjects created with Haploview program (Barrett et al., 2005). Each square representing a pairwise comparison between two SNPs, dark red color indicates high value of D', up to a maximum of 1. The thick black triangle represents a haploblock i.e. region of high LD between the SNPs. Block 1 includes markers from intron 3 of *CREB1*.

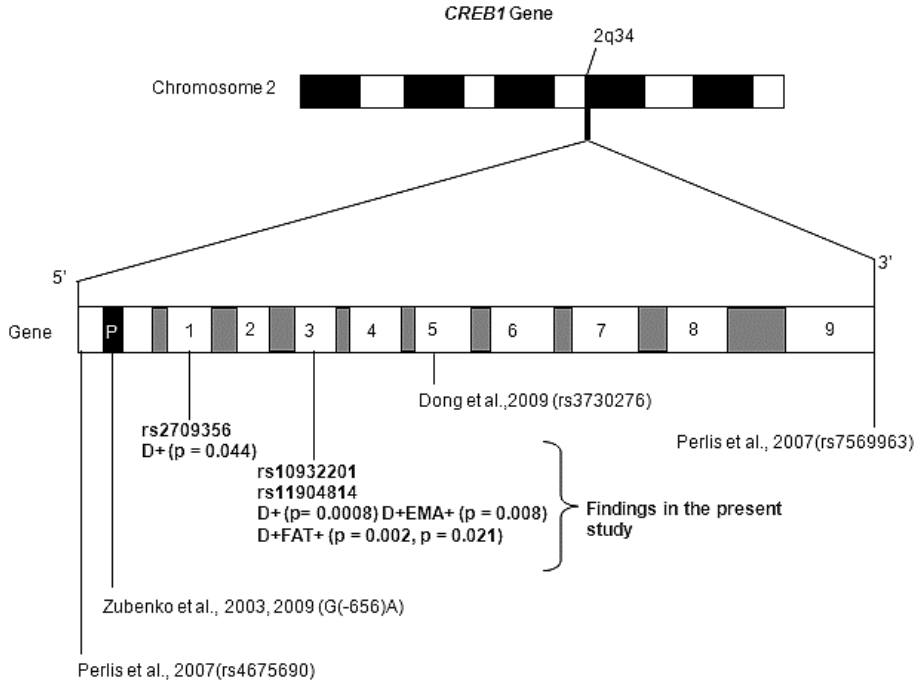


Figure 12. Schematic presentation of the *CREB1* gene. The 5' promoter region (P), 9 introns, and exons (grey blocks) are presented in their approximate locations. SNPs studied from the *CREB1* gene in the present thesis, are highlighted with bold face. The figure also summarizes previous evidence for association of the *CREB1* gene with mood disorders.

In addition, nominal evidence of association between rs3738401 at the 5' end of *DISC1* and depression in females was found (P=0.023, OR=1.30). The non-synonymous SNP rs6265 on exon 2 of *BDNF* leads to an amino acid substitution from valine to methionine at codon 66 (Val66Met), which associates with a decreased distribution of BDNF in neuronal dendrites (Egan *et al.*, 2003). In the present study, a nominal association of allele 'C' (Val66) with depression accompanied by fatigue in females was observed (P=0.027; OR=0.66). Association studies have repeatedly found the involvement of *BDNF* polymorphism (Val66Met) in psychiatric diseases. In other population-based studies, an association of the Val66 (Valine) allele with bipolar disorder and with MDD (and its related personality traits) was also observed (Neves-Pereira *et al.*, 2002, Sklar *et al.*, 2002, Sen *et al.*, 2003, Green *et al.*, 2006). Another variant of *BDNF* from promoter region, rs1491850, also showed nominal evidence of association with depression in females (P=0.015, OR=0.76). Furthermore, no significant evidence of association was observed for the *NTRK2* gene, which is involved in neuronal development and long term memory formation, and supports the stress-induced deficits in the hippocampus which can be

noted in mood disorders (Pittenger and Duman, 2008). The *NTRK2* gene has a complex genomic organization, spanning 350 kb and containing multiple splicing isoforms within the 19 exons. Therefore, it is possible that there are no causative risk variants for depression and disturbed sleep in any of the SNPs tested, thus these variants remain to be found in *NTRK2*.

Amongst the genes from the HPA-axis, nominal evidence of association for the intronic variant rs173365 of *CRHR1* with depression accompanied by early morning awakenings in females was found (P=0.041, OR=1.36, associating allele 'A'). The same allele has also been associated with adult depression arising from childhood abuse (Bradley *et al.*, 2008), suggesting that dysregulation of the HPA-axis is one of the major neuroendocrine abnormalities in depression.

In conclusion, these results support the involvement of candidate genes from serotonergic, glutamatergic, neuralplasticity, and HPA-axis systems in the genetic background of depression and disturbed sleep. These results confirm the genetic heterogeneity of depression and provide evidence for partially separate genetic risk factors among males and females.

5.2 Systematic analyses of circadian genes reveals association of *TIMELESS* with depression and disturbed sleep (II)

The first study of this thesis revealed that gender-dependent and symptom-specific differences may be found in the genetic background of depression and disturbed sleep. The genetic variants which associate with depression also associate with features of disturbed sleep, such as early morning awakenings and fatigue. In study II, this hypothesis was expanded to include genes from the circadian system that could be associated with depression and signs of disturbed sleep. Shortage of light may trigger the seasonal fluctuation which is common in a number of patients with mood disorder (Shin *et al.*, 2005, Grimaldi *et al.*, 2009), and mutations in circadian genes are linked with the metabolic syndrome (Turek, 2008). Therefore, whether the same genes of the circadian system would associate with seasonal variations in metabolic or mental functions was also examined. The associations of a total of 113 SNPs from 18 genes of the circadian system in 1654 subjects from the Health 2000 cohort were investigated.

Statistically strongest evidence was found for allelic variants of the *TIMELESS* gene in both females and males. Of the four SNPs of *TIMELESS* examined, rs7486220 in the intron 1 region showed the strongest association with the minor allele 'A', which increases the risk for depression with fatigue (P=0.000099, OR=1.66) in females (Table 16). This finding survived the correction for multiple testing (Permutation-based corrected empirical P=0.0056, Bonferroni corrected P=0.033). In males, the SNP rs1082214 in the promoter region associated with depression was accompanied by early morning awakenings (D+EMA+) with associating minor allele 'T' (P=0.0009, OR=2.7, Permutation-based corrected empirical P=0.0374, Bonferroni corrected P=0.22). In addition, this allele also associated to D-EMA+ in the second sample set in males (Genmets (D-) samples) (P=0.038, OR=1.52) and D-FAT+ (P=0.0016, OR=1.79). Thus, although the 'T' allele was associated with "depression-free" samples, this allele might increase the risk for symptoms of disturbed sleep like early morning awakenings and fatigue, and in this way it may also impact on risk for depressive disorder.

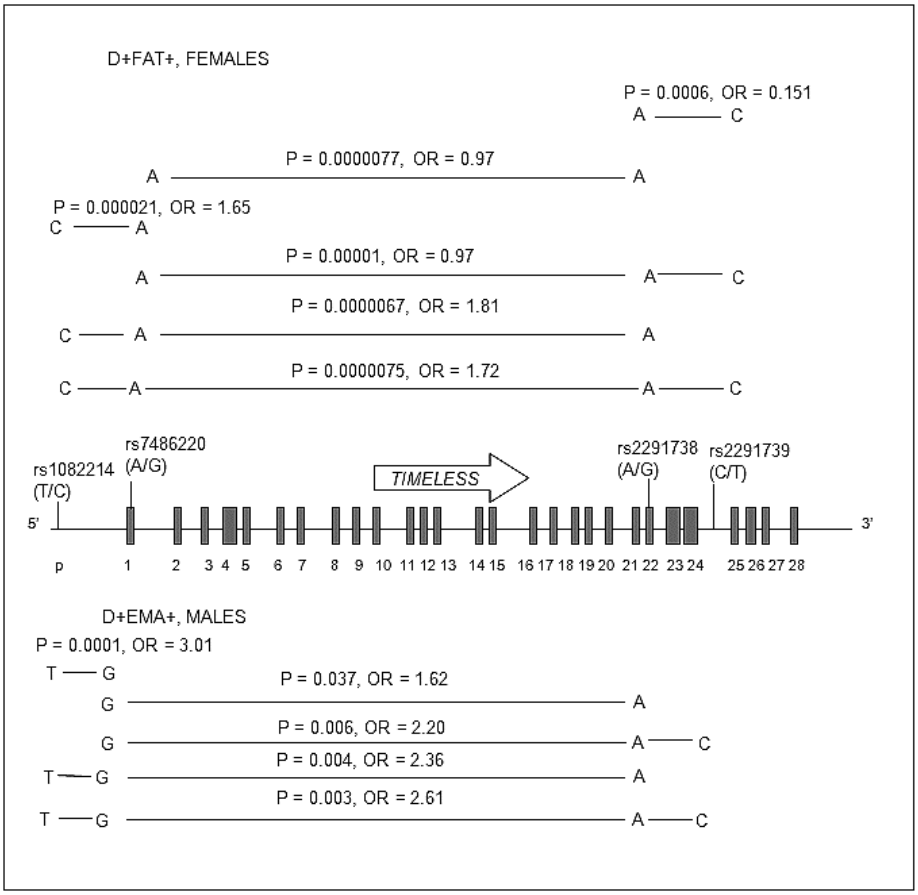
Table 16. The association results of genes from the circadian system with depression and disturbed sleep in females and males.

Chr	*Gene*	*SNP*	*Alleles*	*D+*		*D+EMA+*		*D+FAT+*		*┼MAF (D+)*				*┼MAF (D-)*	*Gender*
				OR	*P-value*	*OR*	*P-value*	*OR*	*P-value*	*EMA-FAT-*	*EMA+FAT+*	*EMA+FAT-*	*EMA-FAT+*	*EMA-FAT-*	
12	*TIMELESS*	rs2291739	T/C	1.21	0.072	1.31	0.060	1.51	**0.0004**	0.3	0.47	0.3	0.44	0.36	*Females*
		rs2291738	G/A	1.22	0.055	1.26	0.110	1.52	**0.0005**	0.37	0.46	0.33	0.47	0.43	
		rs7486220	G/A	1.34	**0.009**	1.55	**0.003**	1.66	**0.000099***	0.26	0.46	0.23	0.40	0.32	
		rs1082214	C/T	0.60	**0.030**	0.33	**0.012**	0.66	0.126	0.03	0.02	0.03	0.06	0.07	
11	*ARNTL*	rs1982350	A/G	1.24	**0.033**	1.09	0.547	1.27	**0.04**	0.48	0.47	0.46	0.44	0.45	*Females*
		rs969485	A/G	0.81	0.121	0.90	0.587	0.70	**0.026**	0.17	0.18	0.4	0.17	0.23	
15	*RORA*	rs4774370	T/C	0.75	**0.045**	0.85	0.449	0.78	0.122	0.11	0.18	0.16	0.17	0.21	*Females*
		rs4774388	A/G	0.76	0.093	0.68	0.128	0.61	**0.010**	0.19	0.10	0.1	0.07	0.14	
9	*NFIL3*	rs1619450	T/C	0.64	**0.022**	0.76	0.316	0.59	**0.017**	0.08	0.09	0.06	0.05	0.11	*Females*
		rs10991925	A/G	1.31	**0.033**	1.06	0.773	1.19	0.227	0.25	0.17	0.3	0.24	0.19	
22	*CSNK1E*	rs135745	C/G	1.17	0.129	1.27	0.107	1.34	**0.015**	0.41	0.46	0.4	0.48	0.45	*Females*
11	*CRY2*	rs10838524	A/G	1.24	**0.038**	1.45	**0.010**	1.22	0.104	0.48	0.45	0.3	0.49	0.46	*Females*
12	*TIMELESS*	rs1082214	C/T	1.56	0.067	2.70	**0.0009***	1.72	**0.037**	0.03	0.15	-	0.03	0.06	*Males*
12	*ARNTL2*	rs4964060	G/A	0.79	0.138	0.73	0.143	0.68	**0.028**	0.4	0.36	-	0.34	0.43	*Males*
		rs7304939	C/T	0.6	0.073	0.54	0.133	0.46	**0.023**	0.15	0.05	-	0.06	0.10	
11	*ARNTL*	rs2290036	T/C	1.70	**0.010**	1.88	**0.014**	1.57	**0.041**	0.2	0.16	-	0.12	0.09	*Males*
2	*NPAS2*	rs12712083	A/G	0.78	0.091	0.87	0.464	0.72	**0.045**	0.4	0.39	-	0.35	0.44	*Males*
15	*TIPIN*	rs2063690	C/G	1.37	0.165	1.72	0.059	1.71	**0.037**	0.03	0.13	-	0.09	0.07	*Males*
17	*PER1*	rs885747	C/G	1.04	0.787	0.66	**0.040**	0.93	0.705	0.4	0.40	-	0.45	0.49	*Males*

Chr: Chromosome. Alleles: Major/Minor. Bold face P-values stand for permutation-based P-values< 0.05. *SNP survived multiple testing correction for association with D+FAT+ in females (rs7486220 Permutation-based corrected empirical P=0.0056, Bonferroni corrected P=0.033), and with D+EMA+ in males (rs1082214 Permutation-based corrected empirical P=0.0374, Bonferroni corrected P=0.22). ┼MAF in non-overlapping group of cases (D+) and controls (D-) based on the presence or absence of EMA or fatigue. D+EMA+FAT- allele frequencies were not examined in this group due to the small number of males (n=3).

Haplotype analyses showed allelic coherence of *TIMELESS* gene in the genetic background of depression and disturbed sleep (Figure 13A). The haplotype C-A-A-C of rs1082214-rs2291738-rs7486220-rs2291739 of the *TIMELESS* gene showed strongest association with D+FAT+ (P=0.0000075, OR=1.72) in females, whereas the haplotype T-G-A-C associated with D+EMA+ (P=0.003, OR=2.61) in males. The haploblock and LD pattern of genotyped markers of *TIMELESS* gene is presented in (Figure 13B).

A)



B)

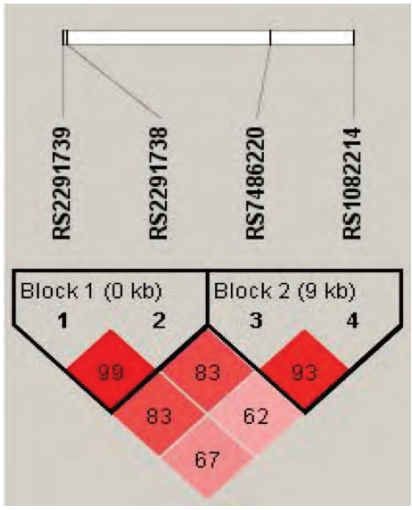


Figure 13. Schematic presentation of the *TIMELESS* gene. Figure 13 A) demonstrates genomic structure and approximate positions of genotyped SNPs within the gene and its promoter region. SNP rs1082214 locates in the promoter region, SNP rs7486220 locates in intron 1 region, SNP rs2291738 locates in intron 22, and SNP rs2291739 in exon 25. Figure 13 A shows 2-SNP, 3-SNP, 4-SNP haplotype association with D+FAT+ in females and D+EMA+ in males respectively. Figure 13 B) demonstrates the linkage disequilibrium between the *TIMELESS* SNPs studied in the present thesis. The LD and haploblock structure of 4 SNPs of *TIMELESS* created with Haploview program (Barrett *et al.*, 2005). Each square representing a pairwise comparison between two SNPs, LD (D') is shown as different shadings of red darker color indicating higher values of D', up to a maximum of 1. The thick black triangles represent haploblocks, i.e. regions of high LD between the SNPs. Block 1 include markers from intron 22 and exon 25. Block 2 includes markers from intron 1 and the promoter region.

The major allele 'C' of rs1082214, which was part of the high-risk haplotype for depression with fatigue in females, also associated to mental factor of GSS (GSSf2) (seasonal variation in sleep duration, social activity, mood and energy level) (P=0.016, β=-0.110). Furthermore, females from "depression-free" (Genmets (D-)) samples showed an association with minor allele 'T' of rs1082214 (P=0.036, β=0.123). Thus, major allele 'C' of rs1082214 is involved in the development of seasonal changes in mood.

Earlier studies have indicated evidence for association between SNPs at the circadian gene loci and bipolar disorder, schizophrenia, (Mansour *et al.*, 2006), insomnia, and mania (Shi *et al.*, 2008). The major allele 'G' of the SNP rs2291738, located in intron 22, was shown here to be associated with D+FAT+ (P=0.0005, OR=1.52) in females, and to be one of the alleles which associated with bipolar

disorder patients (Mansour *et al.*, 2006). These results thus confirm the involvement of *TIMELESS* with susceptibility to mood disorder.

TIMELESS is involved in cell survival after DNA damage (Unsal-Kacmaz *et al.*, 2005), and is an essential component in the regulation of the circadian rhythm. It interacts with PER proteins in the cytoplasm and forms a heterodimer complex, thus allowing their entry into the nucleus, where they inhibit the ARNTL-CLOCK and ARNTL-NPAS2 complexes, which induce the transactivation of *PER1* (Sangoram *et al.*, 1998). Therefore, an interaction analysis of other circadian gene variants with the *TIMELESS* variants was performed here. Evidence was found for interaction between *TIMELESS* rs7486220 and rs3027188 of *PER1* in females with D+FAT+ (P=0.008, OR=0.45). In addition, there was evidence in males with D+EMA+ of interaction between rs2291739 of *TIMELESS* and rs1868049 of *ARNTL* (P=0.0006, OR=4.36), rs7486220 of *TIMELESS* and rs4774370 of *RORA* (P=0.003, OR=3.12), and rs1082214 of *TIMELESS* and rs2269457 of *NR1D1* (P=0.003, OR=3.97). These findings on interaction may point to a genetic network that controls elements of the circadian system, which are also involved in the regulation of sleep and mood. Further, there was evidence for interaction of gender with rs7486220 or rs1082214 to D+EMA+ (P=0.0015 and 0.000023, respectively), and D+FAT+ (P=0.005 for both variants). In addition to *TIMELESS*, polymorphisms from *ARNTL*, *CRY2*, *RORA*, *NFIL3*, *CSNK1E*, *ARNTL2*, *NPAS2*, *TIPIN*, and *PER1* indicated associations with depression and sleep-related problems (Table 16), but these findings do not stand up to correction for multiple testing.

The biocomputational analysis for transcription factor binding sites performed here showed a binding site for a chicken ovalbumin upstream promoter transcription factor (COUP-TF) on 3017 bp from the 5' upstream region of *TIMELESS*. COUP-TF is a nuclear receptor and classified as a member of the steroid receptor family, which plays a role in mitigating estrogen-responsive gene expression (Wang *et al.*, 1989, Klinge, 1999). This could relate to a differential regulation of gene expression in males and females, and also to these findings of different susceptibility alleles for the two genders.

Thus, the results in study II indicate involvement of *TIMELESS* in the etiology of depression and disturbed sleep and provide evidence for the gender-dependent and symptom-specific differences in the genetic background of depression and disturbed sleep.

5.3 Analyses of shared genetic background behind the regulation of depressive disorder and sleep (III)

In the first two studies of this thesis, variants from a number of candidate genes such as, *TPH2*, *GAD1*, *GRIA3*, *BDNF*, *CRHR1*, and *TIMELESS* were observed to be associated with depression and disturbed sleep. These findings suggest that insufficient or disturbed sleep is a precipitating factor in the initiation of depression in those individuals who also have a genetic vulnerability for depression. Moreover, results from studies I and II suggest that the same genetic factors that regulate mood also regulate features of sleep. Our previous study on 18,631 Finnish twins of same sex demonstrated that sleeping less than 7 hours per night increased the incidence of depressed mood 1.7 fold (Paunio *et al.*, 2009). Therefore, a shared genetic background for the regulation of mood and sleep was hypothesized in study III. Consequently, 23 SNPs were selected from 12 candidate genes that had shown evidence of association ($P<0.05$) with depression and disturbed sleep in studies I and II, and their association with sleep duration was assessed in 3147 healthy individuals from the population-based Health 2000 and FINRISK 2007 study samples.

In the single marker association analyses, six SNPs showed nominal association ($P< 0.05$), and rs687577 within intron 12 of *GRIA3* located on the Xq25 was the only variant that associated with sleep duration in females ($P=0.00001$, permutation-based corrected empirical $P=0.00001$, $\beta=0.27$; Bonferroni corrected $P=0.0052$; $f=0.11$) (Table 17). The association appears to be between the minor allele of *GRIA3* variant rs687577 (allele A) and long sleep in females ($P=0.00001$, permutation-based corrected empirical $P=0.0003$, $OR=1.89$; Bonferroni corrected $P=0.039$, $f=0.18$) (Table 17). The association was significant in all three subsamples (Health 2000, Healthy sleepers sample: permutation-based corrected empirical $P=0.002$ and $\beta=0.24$, Health 2000, Genmets (D-) sample: permutation-based corrected empirical $P=0.004$ and $\beta=0.30$; FINRISK 2007 sample: permutation-based corrected empirical $P=0.04$ and $\beta=0.31$).

Table 17. Single marker association results of sleep duration as well as short and long sleep in females (N=1626) and males (N=1521) from the population-based Health 2000 and FINRISK study 2007 samples.

Chr	*Gene*	*SNPs*	*Alleles*	*β /OR*	*P-values*	*Phenotype*	*Gender*
12	*TIMELESS*	rs2291738	G/A	1.22	0.048	Short sleep	*Males*
12	*TIMELESS*	rs1082214	C/T	1.61	0.026	Long sleep	*Males*
12	*TPH2*	rs12229394	G/A	1.28 1.33	0.024 0.041	Short sleep Long sleep	*Males*
15	*RORA*	rs4774388	A/G	1.42	0.010	Short sleep	*Males*
15	*RORA*	rs4774370	T/C	0.72	0.037	Short sleep	*Females*
17	*SLC6A4*	rs4251417	G/A	0.15	0.027	Sleep duration	*Females*
X	*GRIA3*	**rs687577**	C/A	0.27	**0.00001***	Sleep duration	*Females*
X	*GRIA3*	**rs687577**	C/A	1.89	**0.00001***	Long sleep	*Females*

Chr: Chromosome. Alleles: Major/Minor. *Bold face P-values showed permutation-based corrected and Bonferoni corrected P-values< 0.05, and non-bold P-values showed suggestive association (P< 0.05).

Furthermore, a two-SNP haplotype association in intron 12 of *GRIA3* gene revealed a protective haplotype for depression G-A of rs3848874 and rs687577 (study I), which was found to associate with longer sleep duration (P=0.0011, β=0.24). The distribution of rs687577 genotypes (C/C major/major; C/A major/minor; A/A minor/minor) also differed significantly in relation to sleep duration in females (Figure 14, Table 18).

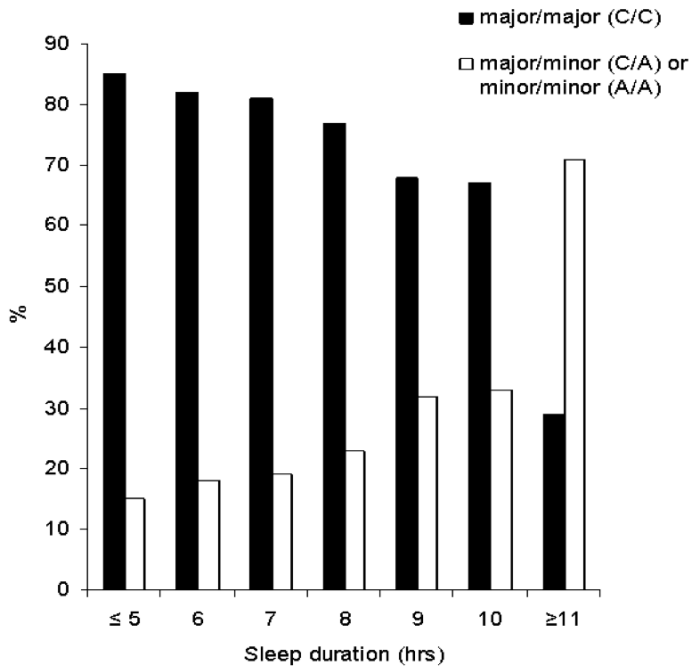


Figure 14. Hours of sleep in females in respect to their genotype at rs687577 from *GRIA3* [Adapted from (Utge *et al.*, 2011)].

Table 18. Genotypes at rs687577 from *GRIA3* in groups of female subjects showing different sleep lengths [Adapted from (Utge *et al.*, 2011)].

Hours of sleep	*MAJOR/MAJOR (C/C)*			*MAJOR/MINOR (C/A) or MINOR/MINOR (A/A)*		
	Number of subjects	*Age (Average±SD)*	*Frequency of C/C*	*Number of subjects*	*Age (Average±SD)*	*Frequency of C/A or A/A*
≤5	29	58.65±11.38	85%	5	60.2±14.72	15%
6	132	53±14.66	82%	29	52.34±16.02	18%
7	450	50.37±12.70	81%	107	47.48±14	19%
8	507	49.66±12.81	77%	150	47.38±13.48	23%
9	114	46.51±16.14	68%	54	44.68±16.40	32%
10	24	52.41±18.68	67%	12	48.75±13.53	33%
≥11	2	45±12.72	29%	5	46.6±15.59	71%

Females who slept for 8 hours or less were more often homozygous (C/C) for the major allele, and a systematic decrease was seen in the proportion of C/C genotype carriers per hour of increase in sleep duration. The results were significant for C/C genotypes even when the group of extreme long-sleepers was excluded (permutation-based corrected empirical P=0.0002, β=0.21). The difference in average sleep duration for C/C genotypes, as compared to C/A or A/A, was observed in all age groups of females younger than 70 years (Figure 15, Table 19). In the previous study (study I), the results showed that major allele 'C' of rs687577 of *GRIA3* was associated with depression (P=0.046, OR=0.70) (Table 14) in females, and here the frequency of this allele was higher in the healthy females with short sleep duration, whereas minor allele 'A' was highly present in the healthy females with longer sleep duration.

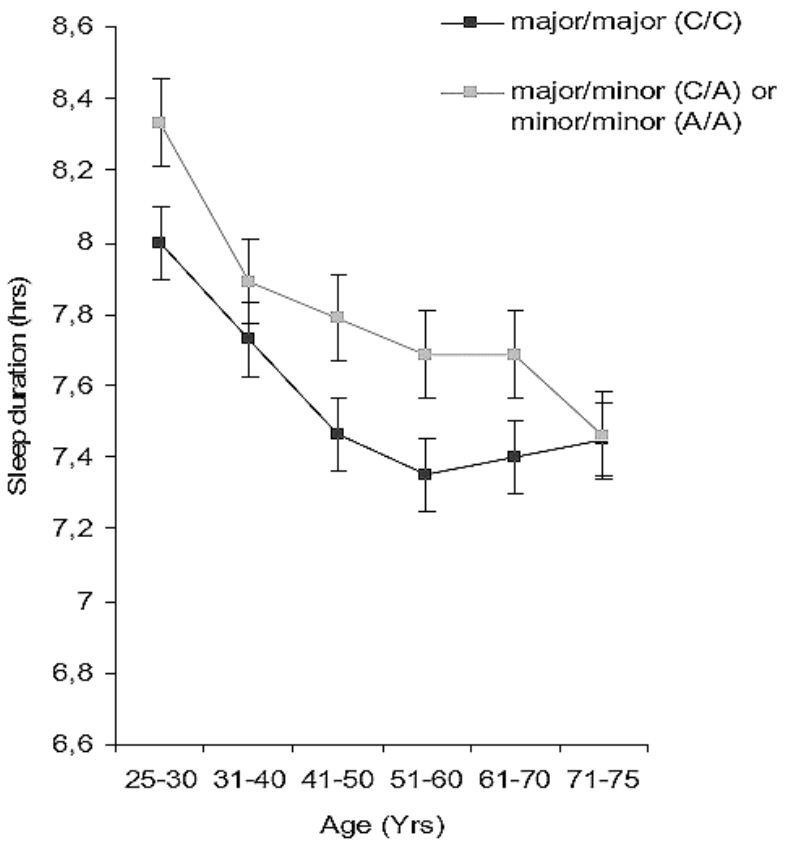


Figure 15. Sleep duration in different age groups of females in respect to their genotype at rs687577 from *GRIA3* [Adapted from (Utge *et al.*, 2011)].

Table 19. Number of female subjects in each age group [Adapted from (Utge *et al.*, 2011)].

Age groups	*MAJOR/MAJOR (C/C)*		*MAJOR/MINOR (C/A) or MINOR/MINOR (A/A)*	
	Number of subjects	*Sleep duration (Average±SD)*	*Number of subjects*	*Sleep duration (Average±SD)*
25-30	23	8±1.04	15	8.33±1.17
31-40	268	7.74±0.87	94	7.89±1.22
41-50	338	7.46±0.88	92	7.79±0.84
51-60	302	7.35±0.96	80	7.68±1.38
61-70	235	7.4±1.04	61	7.68±1.28
71-75	80	7.45±1.13	13	7.46±1.33

The associated region in *GRIA3* was found within intron 12, towards the 3'end of the gene. Previous evidence of association of *GRIA3* with various psychiatric disorders has been identified at the 5' end of the gene (Figure 16). Thus, the results reported here support the probability of an involvement of the 3' region of *GRIA3* in the regulation of sleep duration (Figure 16). In order to further explore the mechanisms for gender-specific association, a transcription factor analysis of *GRIA3* was performed. A binding site for *GATA-3* (*GATA binding protein 3*) was found at the promoter region of the gene (Figure 16): *GATA-3* is a transcription factor with an important role in cellular development and, interestingly, containing one estrogen receptor (ERα) binding site near the 3'end (Usui *et al.*, 2006, Carroll *et al.*, 2006).

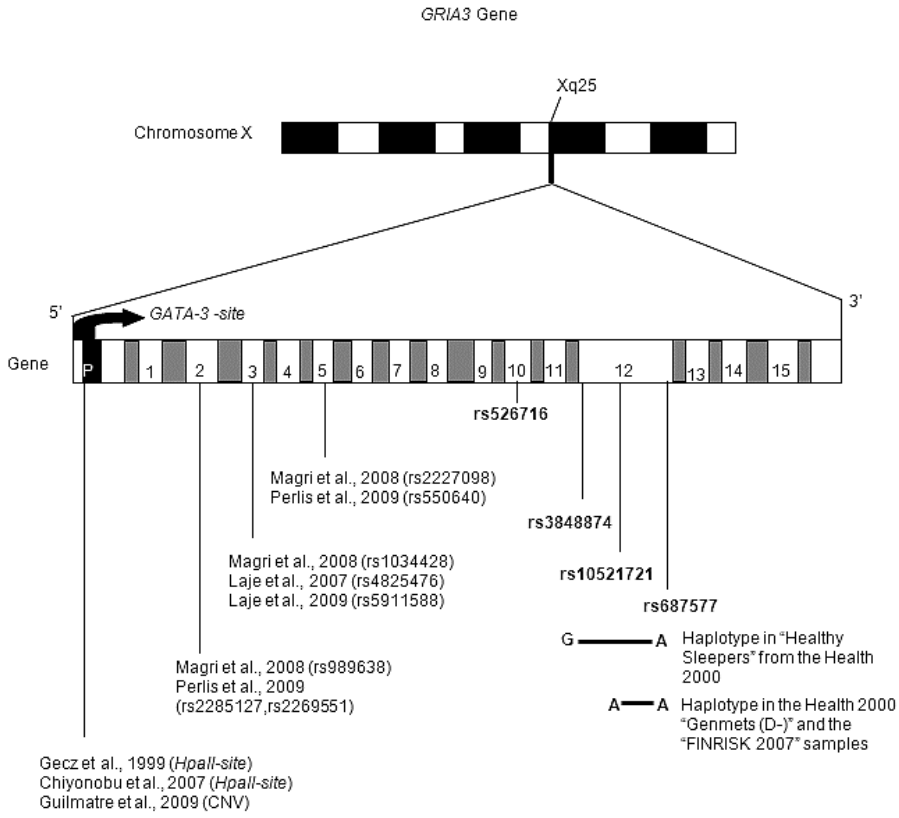


Figure 16. Schematic presentation of the *GRIA3* gene [Figure modified from (Utge *et al.*, 2011)]. The 5'promoter region (P), 15 introns, and 16 exons (grey blocks) are presented in their approximate locations. The bold SNPs indicate those variants that were included in the study, and the core haplotypes that gave evidence for association with sleep duration. A bent arrow indicates the transcription factor-binding site for *GATA-3*, 10 kb upstream (at 122135839 bp) from promoter start (at 122145839 bp). The figure also summarizes previous evidence of association of *GRIA3* with mental retardation and bipolar disorder in females (Gecz *et al.*, 1999), mental retardation in males (Chiyonobu *et al.*, 2007), schizophrenia, mental retardation and autism (Guilmatre *et al.*, 2009), schizophrenia in females (Magri *et al.*, 2008), sexual dysfunction during major depressive disorder (Perlis *et al.*, 2009), and suicidal ideation emerging during citalopram treatment of major depression (Laje *et al.*, 2007, Laje *et al.*, 2009).

In conclusion, an association of the rs687577 variant from *GRIA3* with sleep duration in females was revealed. The same allelic variant of the *GRIA3* gene, which increases the risk for depressive disorders, was also found more often in healthy females with short sleep. The finding points to a shared genetic background in the regulation of sleep and mood as far as involving the mechanism of glutamatergic

neurotransmission, which is, in addition, playing an important role in the induction or maintenance of wakefulness (Manfridi *et al.*, 1999, Wigren *et al.*, 2007).

5.4 Limitations of the studies I-III

Despite these promising results of candidate genes involvement in depression and disturbed sleep, caution must be taken when drawing conclusions. First, the number of individuals with a depression diagnosis was limited and results need to be replicated in other samples and populations. The sample size in studies I and II were small and underpowered to detect associations for variants that increase genetic risk (<1.5 in females and <1.8 in males). The hospital-based MDD cohort study, with prevalence of 0.06 (the average across sexes), also presented that a case-control study of MDD requires a sample size approximately 1.8 times greater (McGuffin *et al.*, 1996). Second, in study I, none of the findings would remain significant if we would subject them to all of the analyses performed. It is noteworthy, however, that detected association with *TIMELESS* (study II) and *GRIA3* (study III) variants sustained after correction for multiple testing. Third, in the present thesis, individuals without information on stress were studied. Therefore, the effect of stressful life events was not studied, although the role of stress in the induction of sleep disturbances or depression is well known. Fourth, in study III, the data on self-reported sleep duration lacks information on possible naps during the day, which could have effect on duration of nighttime sleep, as this was not asked in the Health 2000 and FINNRISK 2007 study surveys. Therefore, future studies with prospective data collection would require a more detailed and comprehensive characterization of sleep regulation and the etiology of depression.

6 CONCLUDING REMARKS AND FUTURE PROSPECTS

Depression is a complex multifactorial disorder which relates to a number of genetic and environmental factors. Earlier studies of depression have provided evidence for an imbalance in neurotransmission in various regions of the brain; however, the precise mechanisms as well as the molecular etiology of depression are as yet largely unknown. Further investigation is needed to identify relevant factors that trigger the onset of the disorder. Most of the patients with major depression have deranged sleep patterns, leading to symptoms such as decreased or increased total sleep, frequent nocturnal awakenings, difficulties falling asleep, early morning awakenings, and fatigue. Evidence from previous epidemiological and twin studies has suggested that the regulation mechanisms of mood and sleep are genetically at least partially linked.

In the present thesis, genetic liability factors which would increase the risk of depression were sought by dividing the patients into categories redefined by the presence or absence of symptoms of insomnia, such as early morning awakenings and fatigue. Genetic variations were examined in functionally relevant candidate genes from serotonergic and glutamatergic neurotransmission, neural plasticity, the HPA-axis, and the circadian system. Many of the polymorphisms from genes from these systems were found that may have a role in the etiology of depression and disturbed sleep. The findings also imply that some of the genetic factors which increase a liability to depression may also influence disturbed sleep. Some of the candidate genes, for example *TPH2*, *GAD1* or *CRHR1* in females, displayed an association with depression when the disorder was accompanied by symptoms of disturbed sleep, while other genes, such as *CREB1* in males, associated with depression regardless of the absence or presence of symptoms of disturbed sleep. This systematical analysis of genes from the circadian system revealed an association of the *TIMELESS* gene to depression with presence of early morning awakenings and fatigue. In females, an association was observed between the same allelic variants of *TIMELESS*, and depression, accompanying fatigue and seasonal fluctuation of mood; while in males another allelic form of the gene showed an increased risk for depression with early morning awakenings. Hence, genetic abnormalities in the circadian clockwork have a potentially great impact on an individual's health. These results also support the heterogeneity of depressive disorder in both a symptom and a gender-specific manner. An allelic variant of *GRIA3* was also identified to associate both with depressive disorder in females and short sleep duration in healthy females in independent population-based samples. Thus, some of the genetic mechanisms underlying the regulation of sleep duration also contribute to a risk of depressive

disorder. Consequently, these results provide evidence of a shared genetic background of sleep and mood.

Identification of genetic variants which predispose to depression and disturbed sleep encourage several avenues for future work. In this thesis, a hypothesis driven candidate gene approach was used. The studied genes were selected due to their role in the pathophysiology of disease and previous evidence connecting them with the regulation of mood. However, when designing a study for heritable disorders, relatively large sample sizes are needed to gain power to detect common variants of smaller effect. The genetic studies have now entered in a new era of genome-wide association studies (GWAS). The key feature of the success of GWAS has been its feasibility to examine large numbers of unrelated individuals by data on 500,000 to 1,000,000 SNPs across the human genome (Raizen and Wu, 2011). Those analyses enable a hypothesis-free search for potential new susceptibility genes. However, due to the high number of statistical tests performed, association is considered significant only with nominal P values of 10^{-8} and with replication of findings in independent cohorts, in order to exclude false-positive results (Sullivan *et al.*, 2009). GWAS is best suited to reveal common genetic variants with small effects for complex genetic disorders. The largest GWAS for MDD to date, the MDD2000+ study, comprised 2431 cases and 3673 screened controls (Wray *et al.*, 2010). It is, however, estimated that sample sizes 1.8- to 2.4-fold greater would be needed for association studies of MDD (Wray *et al.*, 2010). To date, very few GWAS have a targeted genetic basis of sleep disorders. One example is restless legs syndrome (RLS) (Stefansson *et al.*, 2007, Winkelmann *et al.*, 2007, Winkelmann *et al.*, 2011). Clearly many more studies using this approach are needed to study both sleep problems, and those related to depression.

GWAS conducted on complex genetic disorders such as schizophrenia have revealed that the genetic component of those diseases also comprises rare variants with large effects, missed by GWAS (Manolio *et al.*, 2009, Cirulli and Goldstein, 2010). Therefore, future studies will be required to identify rare variants with larger effects. Sequencing all the coding region of the genome (exome) and scanning the mutations that has changed sequence of protein, or sequencing the entire human genome, by using next generation-sequencing technology will help for identification of rare genetic variants and mutations causing human diseases (Ng *et al.*, 2010). Furthermore, sleep is conserved in many model organisms, such as flies, fish, and mice, which could be used for studies focusing on function of the candidate genes revealed by genomic association studies. Thus, combining signals from functional genetic studies in animal models with association studies in humans may lead to a better understanding of mechanisms underlying disease pathogenesis.

Given all of the above, while the search for causal genetic variants to diseases continues, there is also increasing interest in pharmacogenetics. Such studies have, for example, provided information about how antidepressants act differently for people with different genetic backgrounds (Holsboer, 2008). Eventually, advances in understanding the genetic risk and identifying actual disease-predisposing variants will pave the way toward better strategies for treating depression and sleep problems.

7 ACKNOWLEDGEMENTS

This study was carried out during the years 2007-2011 at the Public Health Genomics Unit, National Institute for Health and Welfare, THL (formerly Department of Molecular Medicine, National Public Health Institute, KTL) and Institute for Molecular Medicine Finland FIMM. Also, Institute of Biomedicine, Department of Physiology, University of Helsinki, and Department of Psychiatry, Helsinki University Central Hospital, were involved.

This work could not have been completed without the help and support from a large number of people, to whom I offer my deep and sincere thanks:

Director General of THL Pekka Puska, the Director of Public Health Genomics Unit Anu Jalanko, and the Director of FIMM Olli Kallioniemi for providing all the necessary facilities and resources to conduct research work.

All of the participating subjects of this study, all the field workers and clinicians who have participated in the sample collection and diagnostic assessment.

I would like to acknowledge this work has been financially supported by the European Union Marie Curie Fellowship (MCRTN-CT-2004-512362), and grants from European Union (LSHM-CT-2005-518189), Helsinki University Central Hospital (EVO, TYH6254), Centre for International Mobility (CIMO), and Biomedicum Helsinki Foundation.

My supervisors, Professor Tiina Paunio and Adjunct Professor Tarja Porkka-Heiskanen (Stenberg) provided tremendous support. I gladly take this opportunity to thank Professor Tiina Paunio; you were the one who introduced me to genetic aspects of complex diseases. Your enthusiasm, constructive ideas, guide for the constant help rendered for successful completion of this Project. I am also grateful to her to act as the custos of the dissertation. I feel privileged to have worked with a world-class sleep scientist, respected Adjunct Professor Tarja Porkka-Heiskanen (Stenberg) at the Sleep Team Helsinki. In your laboratory I learnt basically everything that I now know about sleep. Together, the both of you were the real driving force for this thesis, without your valuable guidance I would have never accomplished this work.

Professor Phyllis C. Zee graciously accepted the role of Opponent in my thesis defense. I take this opportunity to express my humble and deep sense of gratitude to my thesis reviewers Professor Björn Appelberg and Adjunct Professor Maija Wess-

man. Your constructive remarks, suggestions were a great source to improve the research work presented in this thesis. I wish to thank Earnst Mecke and Jennifer Rowland for English proofreading.

All of the collaborators and co-authors gave excellent contributions for the projects making up my thesis: Professors Markus Perola, Veikko Salomaa, deceased Academician Leena Peltonen-Palotie for provided the genotype data of the independent set of individuals from Health 2000 and FINRISK study 2007 survey, Professor Sami Pirkola, Adjunct Professors Timo Partonen and Erkki Kronholm had an important role in collecting and characterizing the Health 2000 study subjects, Adjunct Professor Anu Loukola and Emma Sofia Kupriyanova (Nyman) gave a substantial contribution, Pia Soronen, Hanna M. Ollila, and Natalia Gass always had time for me to discuss issues related to my publications, and provided excellent company as colleagues. I thank you all for your support, help and pleasant collaboration.

Leena Laine, Liisa Penttilä, Tuija Koski, Mrja-Liisa Tiainen, Anja Talvitie, and Anne Simola provided generous and kind help in variety of practical matters, my study related issues, and everyday life in Finland.

To the colleagues from the MOOD SLEEP GENE group at the THL, beside those mentioned already, my most heartfelt thanks go to Markus Lagus, Sonja Sulkava, Jukka Alasaari, Antti-Jussi Ämmälä, Marja-Riitta Rautiainen, Auli Toivola, Johanna Salo, and Jenni Kauppinen for excellent company during the years and for friendly atmosphere which made my study much more enjoyable. I sincerely thanks to all the past and present colleagues in THL: especially I would like to acknowledge Olli Pietiläinen for teaching genetic research tools and ready to answer questions those arose, senior scientist Kaisa Silander, Pekka Ellonen, Minttu Jussila, Liisa Arala, Minna Suvela, Virpi Leppä's help for genotyping is highly appreciated. Senior scientist Teppo Varilo, Marika Palo, Marine Largeau, Liisa Tomppo, Juho Wedenoja, Annu Näkki, Nora Pöntynen, Pirkka-Pekka Laurila, Jarkko Soronen, Tea Blom, and in addition to those whom I have forgot to mention for all the good laughs.

A very special thanks goes to all the past and present colleagues in SLEEP TEAM HELSINKI, Andrey Kostin, Wessel van Leeuwen, and Janneke Zant for fabulous company at Marie Curie research trainings and at conferences, I wish to thank Henna-Kaisa Wigren, Kirsi-Marja Rytkönen, Anna Sofia Urrila, Sergey Savelyev, Vilma Aho, Iiris kokkonen, Amy Sanders, Pirjo Saarelainen, Sari Levo-Siitari, and in addition to those whom I have forgot to mention, are thanked for the help and good times during the years.

I am very grateful to have wonderful people around me in day-to-day of my life. I would like to express my gratitude to Praseet and Vandana Poduval, Amarjit and

Sherry Parmar, Shailesh and Rashmi Narawane, Shardul and Sharmila Kulkarni, Rahul Yadav, Nilesh Kanase, Rahul Haware, Krishna Chaithanya, and Biswajyoti Sahu, for your friendship and all of the help I have received.

I am also thankful to my friends who have nothing to do with science, who keep me reminding the life outside Biomedicum. I am grateful for your friendship and unforgettable moments.

Lastly, I owe my deepest gratitude to my family. My parents, Jayraj and Ranjani, and all of the other family members provided me with love, support, and encouragement throughout the years to accomplish this journey. Without the understanding and never-ending support of wife Sofiya, it would have been impossible for me to complete this work, my loving thanks. My baby boy Riyansh; you are the real treasure of my life.

Helsinki, February 2012

Siddheshwar J. Utge

8 ELECTRONIC DATABASE

Databases and program

ABI GENEMAPPER (V.4.0) (http://mvz.berkeley.edu/egl/resources/manuals/GeneMapper_Troubleshooting4.0_.pdf) (Applied Biosystems, Foster City, CA, USA)

ConSite, a platform-independent web resource (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/) (Sandelin *et al.*, 2004)

dbSNP (http://www.ncbi.nlm.nih.gov/snp) (Sherry *et al.*, 2001)

FINRISK study 2007 survey (http://www.ktl.fi/portal/4168) (Peltonen *et al.*, 2008)

Genome browser, EnsEMBL (www.ensembl.org)

HapMap (http://hapmap.ncbi.nlm.nih.gov/) (The International HapMap Consortium, 2003)

Haploview program (V.4.0) (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview) (Barrett *et al.*, 2005)

Health 2000 survey (http://www.terveys2000.fi/indexe.html) (Aromaa and Koskinen, 2004)

MassARRAY Spectro TYPER software (V.2.0 and 4.0) (http://www.sequenom.com) (Sequenom Inc., San Diego, CA, USA)

PLINK software package, web-based version 1.00 and 1.06 (http://pngu.mgh.harvard.edu/~purcell/plink/) (Purcell *et al.*, 2007)

Sequenom's MassARRAY Assay Designer software (V.3.1) (http://cancer-seqbase.uchicago.edu/documents/AssayDesign3.1Guide.pdf) (Sequenom Inc., San Diego, CA, USA)

The Human Genome Project (HGP) (www.ornl.gov/hgmis) (Site sponsored by the U.S. Department of Energy Office of Science, Office of Biological and Environmental Research, Human Genome Program)

The 1000 Genomes Project (http://www.1000genomes.org) (Durbin *et al.*, 2010)

Literature search database

Google scholar (http://scholar.google.com/)

US National Library of Medicine National Institutes of Health (http://www.ncbi.nlm.nih.gov/pubmed/)

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